Studies on the Respiration of *Ashbya gossypii*, a Riboflavin Producing Organism

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A. Biosynthesis of Riboflavin by Microorganisms

The Warburg apparatus has been widely used to study the respiration of cells in order to obtain information about the metabolism of various substances. Of interest is the respiration of the microorganisms that can grow and produce riboflavin on certain media. Among these microorganisms there are some in which growth and riboflavin synthesis can apparently be separated by the use of appropriate media. It would be expected that a study of respiration of these organisms would be of special interest.

Several early workers were interested in the production and identification of a yellow pigment formed by certain microorganisms. The presence of this yellow pigment, which fluoresced under ultraviolet light, was first demonstrated in 1935 by Guillermond, et al., (17) during the growth of an Ascomycete, Eremothecium ashbyii (E. ashbyii). Another organism, Ashbya gossypii (A. gossypii), produced this same pigment, but in much smaller amounts. Rat nutrition studies by Raffy (41) showed that this pigment was identical to riboflavin. Mirimanoff and Raffy (29) concentrated this pigment by repeated extractions and elutions and Raffy (42) showed that its fluorescence spectrum was almost identical to that of riboflavin.

Although Guillermond obtained only small amounts of riboflavin from A. gossypii, Wickerham, et al., (57), using an orange-yellow

variant, were able to produce 38 milligrams per liter after 8 days on an aerated cerelose-yeast extract medium incubated at 26 - 28° C. Ritter (44) separated E. ashbyii into two strains, a white one and a yellow one, and found that the yellow one produced much more riboflavin than did the white.

Riboflavin synthesis has since been demonstrated in the following organisms: Clostridium acetobutylicum (59), Mycobacterium smegmatis (24), Aspergillus flavus (36), Penicillium chrysogenum (49), and species of Candida (51) and Fusarium (30).

The precise conditions required for the growth and riboflavin production of E. ashbyii were first studied by Schopfer (46). He found that as far as riboflavin production was concerned glucose was a better carbohydrate source than maltose, sucrose or levulose. The nitrogen source and the kinds of amino acids present were found to be important, more riboflavin being produced on a peptone medium than on any other natural animal or vegetable medium tried. Later work by Schopfer and Guilloud (47) showed that arginine and leucine could replace peptone as far as riboflavin production was concerned. The three vitamins- biotin, thiamine, and meso-inositol- were found to be essential factors for growth. Prior to this time the work of Fries (14) and Kogl and Fries (19) had shown that these same three vitamins were required for the growth of A. gossypii. At least, they postulated that biotin was required and that thiamine and inositol acted as complementary factors to amplify the action of biotin. Dikanshaya (11) later reported that biotin and inositol, but not thiamine, were required for growth but not for riboflavin production.

Chin (8) using various carbon sources on E. Ashbyii, reported that on a synthetic medium the best sources of carbon were glycerol, glucose and sucrose, with ethyl alcohol next, and that glycol, xylose, and mannitol were not good sources of carbon. As nitrogen sources, peptone, alanine, asparagine, glutamine and ammonium sulfate were good, but tryptophan, tyrosine, cystine, cysteine, histidine and urea were not.

Dikanshaya reported that E. ashbyii grew better on a glucose-salt medium with biotin, inositol, thiamine or casein hydrolysate than on a medium with casein autolysate, peptone, and a combination of leucine, arginine, and histidine or asparagine. The latter medium was made more active by the addition of some casein hydrolysate or certain nitrogen compounds but this more active growth favored lower rather than higher yields of riboflavin. Riboflavin production was found to be favored by low vitamin content and sparse mycelial growth and seemed to be particularly active when growth of the organism was slowing down or had stopped.

Chin obtained the best growth at 25 - 30° C. at pH 6.0 to 6.2 in a synthetic medium containing 1.0 per cent glucose, 0.2 per cent K_2HPO_4, 0.1 per cent $MgSO_4.7H_2O$, 0.1 per cent NaCl, 5.0 per cent rice germ extract, and 5.0 per cent of a nitrogen source. Renaud and Lachaux (43) obtained their largest yield of riboflavin in 24 days on a medium containing 1.2 per cent peptone and 3.0 per cent glucose.

Yaw (60) reported that production of riboflavin by E. ashbyii was increased by the addition of methionine and L- histidine.

Stephens (48), using a chromatographic separation of peptone, was able to show that some fractions of peptone, when added to a medium, would give good growth of A. gossypii with riboflavin formation, whereas

other fractions would give growth with little or no riboflavin formation. He found that a medium containing glutamic acid and arginine monohydrochloride as the only nitrogen sources gave very good growth but very slight riboflavin production. When glycine, leucine, or leucine and histidine were added to this medium, however, noticeable quantities of riboflavin were produced. Further work showed that when a peptone solution was treated with permutit before its use for growing cells no riboflavin was produced. A paper chromatographic analysis of the permutit elution showed the presence of several basic amino acids. A medium prepared with these basic amino acids as the source of nitrogen gave good growth but only slight fluorescence. A water extract of cells of A. gossypii, grown on peptone and yeast extract medium, added to new medium stimulated riboflavin production in the presence of glycine but had no effect in the absence of glycine.

Since the commercial source of riboflavin for several years has been from microorganisms several workers have been interested in studying the growth of these organisms and their riboflavin production on cheap carbon and nitrogen sources.

Desieve (9) found that glucose and unrefined materials containing a large proportion of glucose were good sources of carbohydrate whereas alcohols, such as ethyl alcohol and glycerol, were not. He reported that yeast water and beer worts were good sources of nitrogen but that cystine in concentrations as low as 0.001 per cent reduced the growth of E. ashbyii and inhibited the formation of riboflavin.

A patent issued to Rudert (45) called for the substitution of a metabolizable lipid such as corn oil or lecithin for the carbohydrate

source. Phelps (32) showed that the addition of fatty acid glycerides increased the yield of riboflavin.

Piersma (33) showed that E. ashbyii, grown on a liquid medium composed of 1.5 - 2.0 per cent malt extract, 1.0 - 5.0 per cent animal protein (such as tankage, fish meal, liver, pancreas, spleen, kidney, etc.) and 0.25 - 1.5 per cent of a carbohydrate source (such as molasses or corn syrup) produced 150 - 200 gamma of riboflavin per milliliter of medium when grown with continuous aeration. Piersma's medium was modified by Phelps to the extent that the animal protein was replaced with solubilized casein. Rudert reported that the length of time for fermentation depended upon the incubation temperature, the per cent of inoculant, and the nutrient content of the mash, but that fermentation was usually complete in 50 - 90 hours at 30° C. The pH suitable for fermentation was reported to be between 4.5 and 9.1.

Factors affecting the commercial production of riboflavin by A. gossypii were studied by Tanner, et al., (50) who found that satisfactory nitrogen sources consisted either of crude animal proteinaceous material or of combinations of corn steep liquor with animal materials such as animal stick liquor, tankage, and meat scraps. As the source of the fermentable sugar, glucose, maltose, and sucrose were assimilated but pentoses were not. Their culture was maintained on agar slants containing 1.0 per cent glucose, 0.5 per cent peptone, 0.3 per cent yeast extract, 0.3 per cent malt extract, and 1.8 per cent agar. Konenklijke (20), using partially hydrolysed assimilable carbohydrates and proteins from both animal and vegetable sources, obtained yields of 600 gamma of riboflavin per milliliter of broth.

Fridham, et al., (39) found that aerobic submerged fermentation gave good yeilds of riboflavin at low costs. Tanner, et al., found that for commercial production of riboflavin the best temperature was between 26 and 28° C., and that the best pH was between 6.0 and 7.0. Young inoculum and a minimum time of sterilization were also important factors.

Along with the increased use of A. gossypii as a commercial source of riboflavin came an interest in determining how and why this organism produced riboflavin in excess of its own needs. Various workers investigated precursors of riboflavin and used radioactive compounds to discover the pathway of riboflavin formation.

MacLaren (23) found that xanthine and certain other purines stimulated riboflavin formation in E. ashbyii, whereas pyrimidines inhibited it. He postulated a possible purine pathway of riboflavin formation. Adenine was found to be a good precursor of riboflavin by McNutt (25) who showed that it was incorporated principally into the 6,7-dimethyl isoalloxazine portion of the molecule.

Plaut (34, 35) showed that C-14 labeled formate, bicarbonate, C-1-14 or C-2-14 labeled acetate, or C-1 or C-2 labeled glycine were all incorporated into various positions in the riboflavin molecule produced by A. gossypii. Identical results were obtained with Glucose-1-14 or Glucose-6-14 which suggested that both ends of the molecule yielded similar two carbon fragments which were employed in building up the first ring of the riboflavin molecule.

Much of the present understanding of metabolism is due to data obtained from the use of the Warburg apparatus. This gives a convenient means of study of metabolic transformations which may occur as a part of

the overall picture of metabolism. It was, therefore, logical that Warburg techniques should be applied to the study of the metabolism of A. gossypii.

Nickelsen (26), studying the oxidation of glucose by A. gossypii, found that 0.0001 M cyanide completely inhibited respiration. This indicated that a cytochrome system might be involved. Glucose oxidation was completely inhibited by 0.01 M iodoacetate, which only slightly inhibited the endogenous respiration. Sodium fluoride in concentrations of from 0.01 M to 0.075 M completely inhibited glucose oxidation and reduced the endogenous respiration by 46 to 92 per cent. The iodoacetate and fluoride inhibitions indicated that phosphorylated intermediates might be a part of the oxidation cycle. Nickelson found that riboflavin production did not occur during the early stages of the fermentation when the pH was low and the sugar being rapidly consumed but that it started after the sugar had been consumed and the pH had risen. So it appeared that riboflavin formed after maximum growth had been reached and that its formation was related to the transformation of cellular constituents synthesised by the growing mycelium rather than by a direct synthesis from compounds added to the medium. Washed mycelia suspensions would oxidise glucose, sucrose, and maltose, but not lactose. In the early part of the fermentation of glucose considerable ethyl alcohol and traces of acetic acid were formed, but ultimately oxidation proceeded almost completely to carbon dioxide and water. Pyruvic, succinic and acetic acids and ethanol were also oxidised.

Nickelson and Schuler (27) found that when mycelial suspensions of A. gossypii were cultured in the presence of ethanol their ability to

oxidize acetate was increased by as much as fourfold. Such suspensions also oxidized alpha-ketoglutaric, succinic, formic, malic, oxalacetic, and citric acids. Additions of small amounts of alpha-ketoglutaric, succinic, fumaric, or malic acids to suspensions which were oxidizing 0.02 M acetate stimulated oxygen consumption beyond that of additive values from the substrate and added acid. Oxalacetic, pyruvic, and citric acids had no stimulative effects on acetate oxidation. Citric acid was apparently synthesized during acetate oxidation and the amount produced was increased by the presence of oxalacetate. Dried mycelium retained a rather high oxidative activity for succinate and citrate and to a slight extent for some other members of the tricarboxylic acid cycle, but completely lost its activity for acetate and ethanol. No evidence was obtained for a condensation of acetate to succinate during oxidation.

B. Use of the Warburg Apparatus in the Study of Metabolic Processes

Warburg (55) first showed that animal tissues and organs could be prepared in such a way that metabolic processes could be carried on after their removal from the animal body and that this metabolism could be followed quantitatively by means of exchange of gases, usually oxygen and carbon dioxide. The entire apparatus used for the measurement of this exchange of gases is known as the Barcroft-Warburg respirometer and is usually considered to consist of a manometer fitted with a removable sample vessel and a constant temperature water bath equipped with a thermoregulator, stirrer and shaking mechanism. The apparatus has been described in detail by Perkins (31). The most common type of manometer used is referred to as the Warburg or constant volume respirometer.

A circular type of bath described by Lardy, et al., (22) is now the most generally used type. This accomodates more manometers per given unit of space than the rectangular baths and it is equipped with a rotating carriage which allows the manometers to be brought to the operator.

The Warburg vessels may be equipped with one or two sidearms which make it possible to change the chemical environment in the vessel by adding materials to it during the course of the reaction (7).

The errors of measurement were reported by Dickens and Simer (10) and Dixon (12), who estimated the total error inherent in the manometric technique itself to be approximately 2 per cent and considered an overall error of 5 per cent with experimental work to be very good.

Perkins reported that the major limitations of the Warburg were:

1- Inability of the oxygen to diffuse from the gas phase into the liquid phase at a sufficiently rapid rate.

2- Dependence of the rate of gas uptake on the rate of shaking.

3- Insufficient rate of absorption of carbon dioxide by the alkali present.

4- Use of improperly designed flasks which inhibit diffusion of the gas into the liquid.

5- Diffusion of gases through ground glass joints.

The two common methods for measuring oxygen consumption, according to Umbreit, et al., (52) are the direct and the indirect methods. In the direct method the oxygen uptake by living tissues (which also liberate carbon dioxide) is measured by absorbing the carbon dioxide continuously in alkali. This method can be used only when oxygen and carbon dioxide are the only gases exchanged. Under other conditions the indirect method

must be used. In the indirect method two flasks contain the same amount of respiring material but have different fluid volumes. It must be assumed that the same reaction is occurring in both flasks and that the amounts of oxygen consumed and carbon dioxide liberated will be the same in both cases.

The endogenous respiration, or respiration in the absence of a substrate, has frequently been assumed to continue at the same rate after substrate has been added. Barker (3), Douberoff (13) and Van Niel, et al., (54) have shown that endogenous metabolism is frequently inhibited when an external substrate is added. Van Niel (53) stated, therefore, that it would be better to report the actual data for both endogenous respiration and respiration in the presence of a substrate rather than corrected values only.

Among the many applications of Warburg technique the following might be listed: studies on the oxidation of ascorbic acid (4), fatty acids (5), fatty oils (18), and tyrosine (21); respiration of wheat (1) and other cereal grains (37); microanalysis of gases to determine nitrogen fixation (7); determination of glutathione (58) and Coenzyme I (2) in tissues, and arginase (56) in blood; study of bacterial growth (16); enzyme purification (6); studies on photosynthesis in algae (15); and the determination of cozymase (40) and catecholase (28).

II. STATEMENT OF THE PROBLEM

Ashbya gossypii is an important commercial producer of riboflavin. Much is known about the conditions which are required in order to produce large yields of riboflavin, yet little is known about the metabolic processes involved. This study of the respiration of Ashbya gossypii, both under those conditions where it produces riboflavin, and under those where it does not, was undertaken in an effort to find information on the possible metabolic changes associated with the synthesis of riboflavin.

III. EXPERIMENTAL

A. The Apparatus

1. The colorimeter. An Evelyn colorimeter equipped with a light beam galvanometer was used for the turbidity measurements, the turbidity varying inversely as the per cent transmission. The light source was passed through a green filter with a dominant transmitted wavelength of 540 millimicrons. The galvanometer was set at 100 per cent transmission against distilled water and the turbidity measurements of the suspensions were made, in test tubes calibrated for use with the instrument, by measuring their per cent light transmission. Suspensions were diluted to the desired turbidity by the addition of buffer.

2. The shaker. A platform reciprocating shaker with 90 three inch strokes per minute was used to maintain aeration of the organisms during incubation. The removable top of the shaker contained 50 two and one-half inch holes to accomodate 250 ml. Erlenmeyer flasks. This shaker was kept at 28° C. in a constant temperature room.

3. The pH meter. A Beckman Model H-2 line operated pH meter equipped with a Beckman glass indicator electrode for use from 0 to 11 pH, and a saturated calomel reference electrode, was used for all pH measurements. The meter was standardized each time before use against a pH 7.0 buffer, prepared from a Beckman concentrated liquid buffer and a pH 4.6 acetate buffer.

4. The Warburg apparatus. A Precision Scientific Company

circular Warburg apparatus with space for twenty manometers was used according to the procedure described by Umbreit, et al. A bath temperature of 30° + or - 0.01° C. was maintained through all experiments.

The Warburg flasks were prepared in the following manner. To the center well of a Warburg flask was added 0.2 ml. of 20 per cent KOH solution. Filter paper was cut in a 1½ by 3 cm. strip, then folded in accordion pleats and was placed in the center well in order to increase the surface of the alkaline solution exposed to the air in the flask. No substrate was added to the flask when endogenous respiration, or respiration in the absence of substrate, was to be measured. Otherwise, the required volume of substrate was placed in the sidearm. The glass sidearm stopper was then inserted and covered with a rubber policeman in order to avoid leakage through the gas vent. Sufficient buffer was placed in the main body of the flask to make a total liquid volume of 2.2 ml. including the liquid in the sidearm. One milliliter of a prepared cell suspension was then added to the flask and the flask was attached to the manometer. Then the flask was immersed in the constant temperature bath and was allowed to equilibriate by shaking for 10 minutes with the manometer open to the air. At the end of this time the manometer was closed and the initial reading was taken by adjusting the closed arm of the manometer to 250 mm. and reading the open arm of the manometer. The flask was then closed to the air and subsequent readings were taken on the open arm of the manometer at the desired time intervals, the closed arm of the manometer being adjusted to 250 mm. before each such reading.

A thermobarometer, which serves as a control on atmospheric

pressure and temperature changes, was run in the same manner with each set of flasks. This thermobarometer consisted of a Warburg flask and manometer assembly in which the flask contained only 3.0 ml. of pH 6.0 buffer and 0.2 ml. of 20 per cent KOH in the center well. The reading of the thermobarometer was added to or deducted from the readings of the other manometers depending upon whether the thermobarometer reading represented an increase or a decrease.

Each reading, after correction for the thermobarometer, gave the difference in pressure between the interior of the flask and the open air and from it the volume of gas absorbed by the respiring cells was calculated according to the equation

$$x = k \frac{V_g \frac{273}{T} + V_f}{P_o}$$

where x is microliters of gas at standard pressure and temperature; h is the observed change in mm. on the open side of the manometer; V_g is the volume of gas in the flask including connecting tubes down to the level of the manometer fluid; V_f is the volume of all liquids in the flask; k is 0.027, the solubility of oxygen, expressed in milliliters, per milliliter of liquid in the flask at 30° C., when the oxygen is at a pressure of one atmosphere; T is the absolute temperature of the water bath or 303° A.; and P_o is standard pressure expressed in terms of the manometer fluid used. Brodie's solution, which has a specific gravity of 1.033, was used as the manometer fluid so that P_o was 10,000 mm.

The flask volume is determined from the weight and the density of mercury required to fill the flask. Since the fluid volume is constant at 3.2 ml., this value can be subtracted from the total flask

volume to give the gas volume of the flask. For constant conditions, then, the quantity

$$\frac{V_g \frac{273}{T} + V_f}{P_o}$$

remains constant for any one flask and is known as the flask constant. Using the equation

$$x = h\,k$$

where k is the flask constant, the millimeters pressure change can be converted directly to microliters of oxygen absorbed. The volumes and flask constants of the flasks used are given in Table 1.

TABLE 1

VOLUMES AND FLASK CONSTANTS OF THE WARBURG FLASKS USED

Flask Number	Total volume	Gas volume	Flask constant*
	ml.	ml.	
1	19.180	15.980	1.448
2	19.119	15.919	1.443
3	18.356	15.156	1.374
4	19.814	16.614	1.506
5	20.671	17.471	1.583
6	19.946	16.746	1.517
7	18.665	15.465	1.402

(Table continued on following page)

TABLE 1 (continued)

Flask Number	Total volume	Gas volume	Flask constant*
	ml.	ml.	
8	17.568	14.368	1.304
9	19.164	15.964	1.447
10	17.229	14.029	1.273
11	20.989	17.789	1.611
12	19.662	16.462	1.483
13	19.126	15.926	1.444
14	19.602	16.402	1.478
15	20.189	16.989	1.540
16	18.811	15.611	1.416
17	17.232	14.032	1.273
18	21.023	17.823	1.615

*The other values needed for the calculation of the flask constant are: V_f is 3.2 ml., T is 303° A., P_o is 10,000 and is 0.027.

B. Cell Techniques

1. Maintenance of the culture. A culture of A. gossypii, strain NRRL-Y-1056, obtained from the Northern Regional Research Laboratory in Peoria, Illinois, was maintained on agar slants containing 2.0 per cent glucose, 1.0 per cent peptose, 0.5 per cent yeast extract, and 1.8 per cent agar. Every two or three days, transfers were made to new slants which were grown at 28° C. in an incubator room.

2. Production of cells for experimental purposes. The cells were

grown on a basal liquid culture medium prepared by dissolving 0.1 g. KH_2PO_4, 0.1 g. K_2HPO_4, 0.05 g. NaCl, 0.05 g. $MgSO_4.7H_2O$, 2.0 g. glucose, 3.0 mg. inositol, 0.3 mg. thiamine, 2.0 ug. biotin, and approximately 50 mg. of nitrogen from the desired nitrogen source in sufficient distilled water to make 100 ml. of solution. The pH of this medium was adjusted to 6.5 with a 20 per cent KOH solution, except where peptone was used as the nitrogen source. In this case the pH of the medium was approximately 6.5 without further adjustment. The medium was then transferred to 250 ml. Erlenmeyer flasks, 20 ml. being placed in each of duplicate flasks. The flasks were sterilized at 15 lbs. pressure for 20 minutes and they were removed from the autoclave as soon as it had cooled to 212° F.

An inoculum was made from a 2 day old agar slant by adding 10 ml. of water to a slant, scraping the mat of growth from the agar, and breaking it up with a nichrome wire loop. The cells were then centrifuged, washed twice with 10 ml. portions of water and finally made up to a volume of 10 ml. with water, sterile distilled water being used at all times during the preparation of the inoculum. This produced a suspension with a turbidity of between 40 and 60 per cent transmission. One milliliter of this suspension was placed in each of the flasks to be inoculated, inoculations being made in duplicate. The flasks were then placed on the reciprocating shaker and held at 28° C. in the constant temperature room. The flasks were removed from the shaker for measurement when the desired age of cells had been reached.

3. Preparation of the cell suspensions. After the flasks were removed from the shaker the cells from the duplicate flasks were combined,

centrifuged, washed twice with 10 ml. portions of 0.1 M phosphate buffer (either pH 6.0 or pH 8.0) and then homogenized with buffer in a Waring blendor. After homogenization the cells were washed once more with 10 ml. of buffer and the suspension was made up to the desired turbidity by the addition of buffer. The pH 6.0 buffer was prepared by mixing 12 ml. of 0.1 M Na_2HPO_4 (14.21 g/l) with 88 ml. of 0.1 M KH_2PO_4 (13.62 g/l). The pH 8.0 buffer was made by mixing 97 ml. of 0.1 M Na_2HPO_4 with 3 ml. of 0.1 M KH_2PO_4. The buffers were stored in the refrigerator at all times when they were not in use and while they were being used they were placed in a beaker of crushed ice and water.

After each washed, homogenized cell suspension was prepared a 1 ml. portion of the suspension was transferred to the Warburg flasks using a 1 ml. delivery pipette.

In order to determine the dry weight of cells in each suspension a 10 ml. aliquot was filtered with suction onto a previously dried and weighed filter paper. The filtered cells were placed in a drying oven at 100° C. overnight and then weighed. The weight of the cells was calculated by difference.

All cell suspensions used in the following studies were prepared as given above except where otherwise noted.

C. Experiments

Some of the experiments in this study were made to determine variables within the technique which would need to be controlled to obtain optimal conditions for the Warburg runs. Other experiments deal with the effects of various substances in the growth media, as well as in

the Warburg flasks, on respiration.

1. Effect of homogenization of cell suspensions. The growth of A. gossypii occurs in clumps so that it is difficult to prepare homogeneous suspensions. Thus it was decided to study the effects of homogenization using the Waring blendor.

A washed suspension of 24 hour cells grown on the basal medium with 0.172 per cent added peptone was prepared. One aliquot of the suspension was removed before homogenization; the rest of the suspension was homogenized in the blendor. Aliquots were removed from the blendor after 5, 10, 15, and 20 second homogenization periods. Each aliquot was adjusted with pH 6.0 buffer to a transmission of 50 per cent.

Warburg flasks were set up in duplicate to measure the endogenous respiration for each aliquot and were run for 90 minutes in the Warburg at a shaking rate of 115 strokes per minute. The results are given in Table 2.

These results show that not only does a 5 second homogenization period give better duplicate results but it also gives an increase in the respiration. The 10, 15, and 20 second homogenization periods also showed good duplication of results but the respiration was decreased to a large extent.

The increase in respiration of the 5 second homogenization over no homogenization may have been due to the breaking up of clumps of cells or aggregates of cells so that more surface was exposed to the flask medium. The decrease with longer periods of homogenization was perhaps due to destruction of a portion of the cells thus decreasing the numbers of respiring cells. As a result the 5 second period of homogenization

was selected for this treatment of the cell suspensions in other experiments.

TABLE 2

TOTAL MICROLITERS OF OXYGEN TAKEN UP IN 90 MINUTES BY A 24 HOUR PEPTONE CELL SUSPENSION* WITH VARIOUS TIMES OF HOMOGENIZATION

Time of homogenization	Oxygen absorbed Endogenous
sec.	uL
0	84.1
	73.1
5	92.6
	90.4
10	56.4
	53.1
15	47.7
	45.8
20	40.0
	46.7

*Cell suspension was diluted to 50 per cent transmission.

2. Effect of position of the thermobarometer on respiration measurements. It was noted during several early runs that thermobarometer corrections were extremely high at times and seemed to vary somewhat with the position in the bath where a flask was located. Since one thermobarometer serves as a control on temperature and pressure changes for all of the manometers on the Warburg at any one time it seemed advisable to check whether the position of the flask and manometer on the

bath had any effect on the observed readings.

Four flasks were set up, each containing 3.2 ml. of pH 6.0 buffer only. These flasks were attached to the manometers and were placed in the Warburg, one each on the north, south, east, and west positions of the water bath. Readings were taken at various time intervals over a period of 140 minutes. The total changes in volume during this time are shown in Table 3.

During the above run it was noticed that a "dead spot" occurred at the surface of the water bath between the north and west sides of the bath near the heating cell. There appeared to be little effect of stirring in this position which possibly accounted for the higher reading from the manometer located on the west side of the bath. The blades of the stirrer were bent up to give increased circulation which apparently eliminated the "dead spot". After this adjustment of the stirrer another run was made in the same manner. Identical runs to further study the effect of position of the thermobarometer were made in July and October. The total changes in volume that occurred during 140 minutes in the Warburg in the above runs are given in Table 3.

The original results and the results obtained after the adjustment of the stirrer indicate that the condition which caused the high readings was at least partially corrected by the adjustment of the stirrer. In the case of the July run, the room door, which opened on the northwest corner of the Warburg, was open and sunlight was coming into the room when the run was made. No readings of air temperature were made but it may be supposed that the temperature was higher at the north

TABLE 3

TOTAL VOLUME CHANGES OF THERMOBAROMETERS
IN 1h0 MINUTES IN THE WARBURG

Run	Total change* for each position on the bath			
	North	South	East	West
	uL	uL	uL	uL
Before stirrer adjustment	- 0.5	+ 1.0	+ 0.5	+ 6.5
After stirrer adjustment	+ 3.5	+ 2.0	+ 2.5	+ 2.0
July	+12.5	+ 9.5	+ 8.5	+ 7.5
October	+18.0	+17.0	+17.0	+12.0

*Plus readings indicate an increase over the initial reading and minus reading indicates a decrease from the initial reading.

manometer than it was at the other manometers. A check of air temperature was made during the October run. The temperature of the air near the west manometer was one degree lower than that near either of the other manometers, which could account for the lower reading by the thermobarometer in that position. Since air temperature has an effect on the temperature of the apparatus outside the bath, an attempt was made at all times to avoid drafts and direct sunlight which could cause temperature changes in the thermobarometer to vary from position to position on the Warburg.

Subsequent runs were made with the thermobarometer just north of the east side, since this position appeared to give the best results. The thermobarometer was the last manometer to be read in each run so that it made a full circle of the bath each time before its reading was taken.

3. Effect of rate of shaking in the Warburg apparatus on respiration measurements. A suspension of 24 hour cells grown on the basal medium with 0.172 per cent added peptone was prepared to study the effects of rate of shaking of Warburg flasks on the respiration measurements, since the rate of shaking is one of the major limiting factors in the ability of oxygen to diffuse from the gas to the liquid phase of the flask medium. The suspension was diluted with pH 6.0 buffer to a 20 per cent transmission and aliquots were run in the Warburg at three different rates of shaking- 100, 118, and 132 strokes per minute- in order to determine whether there were any differences in the amount of oxygen absorbed by the cells. For each shaking rate four samples were run, two of which contained 0.5 ml. of 0.1 M glucose as substrate, and two of which contained no substrate to determine endogenous respiration. Aliquots of the suspension were run immediately after preparation of the suspension at the 100 stroke per minute rate. In a similar manner, aliquots of the suspension were run at the 118 and 132 stroke per minute rates. Since it takes about one hour to make a run, the suspensions were stored at 10° C. in a refrigerator for one and two hours before the 118 and 132 stroke per minute rates, respectively. In each case the total volume of oxygen was recorded after 50 minutes. The results are given in Table 4.

These results show that approximately the same respiration measurements are obtained with all three different rates of shaking. Obviously, since there is no increase in oxygen uptake with increased rate of shaking, the rate of diffusion of oxygen between the gas and liquid phases of the flask medium is not a limiting factor at these rates.

TABLE 4

RESPIRATION MEASUREMENTS AT VARIOUS RATES OF SHAKING A CELL SUSPENSION*

Strokes per minute	Oxygen absorbed/ 50 min.		
	Endogenous	Substrate**	Substrate minus Endogenous Ave.
	uL	uL	uL
100	11.7	99.3	
	19.5	69.7	68.9
118	33.6	93.9	
	30.9	89.0	59.2
132	19.3	69.2	
	31.3	103.2	60.8

*Suspension prepared from 24 hour peptone cells and diluted to 20 per cent transmission.
**Substrate consisted of 0.5 ml. of 0.1M. glucose per Warburg flask.

Any one of the rates of shaking, then, should be satisfactory for the particular working conditions involved. The 118 stroke per minute rate was arbitrarily chosen for use in the subsequent experiments.

4. Effect of the amount of inoculum, in the growing of cells used for suspensions, on respiration measurements. In order to study the effect of the amount of inoculum, duplicate flasks, containing the basal medium with 0.172 per cent added peptone as the nitrogen source, were inoculated with 0.1, 0.5, 1.0 and 1.5 ml., respectively, of a cell suspension prepared in the usual manner. After 24 hours incubation the contents of duplicate flasks were pooled and diluted to 40 ml. with distilled water, after which pH and turbidity measurements were made. The results obtained are given in Table 5.

TABLE 5

THE EFFECT OF AMOUNT OF INOCULUM ON GROWTH AND pH IN PEPTONE MEDIUM

Amount of Inoculum	Transmission	pH
ml.	per cent	
0.1	51.5	6.30
0.5	41.0	6.35
1.0	34.5	6.40
1.5	35.5	6.30

The above cells were washed in the usual manner and diluted with pH 6.0 buffer to prepare cell suspensions with a turbidity of 50 per cent transmission. A 10 ml. aliquot of each of the 4 suspensions was dried at 78° C., and weighed, in order to determine the dry weight of each suspension. Respiration measurements were made on these suspensions, half of the samples in each case being run with 0.5 ml. of 0.1 M. glucose as substrate, and the other half being run without substrate to determine the endogenous respiration. All samples were run in the Warburg for 140 minutes and the oxygen uptake in each case was calculated as microliters of oxygen per milligram of dry weight of cell suspension in the Warburg flask. The results obtained are shown in Table 6.

The results in Table 6 show that the greatest respiration with glucose occurs in the cells originally started with 0.1 ml. of inoculum. The growth in this case, however, is less than with the larger amounts of inoculum, as can be seen from the data in Table 5. This increased

TABLE 6

THE EFFECT OF AMOUNT OF INOCULUM ON RESPIRATION MEASUREMENTS OF CELL SUSPENSIONS*

Amount of Inoculum	Oxygen absorbed/ mg. of cells/ 1½0 min.			
	Endogenous	Substrate**	Substrate minus Endogenous Ave.	Dry Weight
	uL	uL	uL	mg/ml
0.1	33.0	94.2		
	32.2	83.8	56.4	1.58
0.5	43.3	70.4		
	35.7	76.3	38.9	1.61
1.0	21.3	60.8		
	20.4	83.2	51.2	1.42
1.5	23.4	84.9		
	28.6	60.9	46.9	1.40

* Suspensions prepared from 24 hour peptone cells and diluted to 50 per cent transmission.

**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.

respiration with the 0.1 ml. inoculum may be due to the cells still being in the logarithmic phase of growth at the end of 24 hours, where metabolism is being carried out at an extremely rapid rate and the need for glucose is greatest. However, because of the decreased growth obtained with this amount of inoculum, 0.1 ml. was considered to be unsatisfactory. The highest respiration among the three higher amounts of inoculum was obtained with the 1.0 ml. amount of inoculum. This amount of inoculum was considered to be satisfactory and was used in all subsequent experiments.

5. Effect of concentration of the cell suspension in the Warburg flasks. A washed suspension of 48 hour cells grown on the basal medium

with 0.172 per cent added peptone was prepared to study the effect of the concentration of the cell suspension on respiration measurements. This suspension was divided into three portions which were diluted with pH 6.0 buffer to make suspensions with transmissions of 10, 20, and 35 per cent. A 5 ml. aliquot of the 10 per cent transmission suspension and 10 ml. aliquots of the 20 and 35 per cent transmission suspensions were used to determine the dry weight of each suspension. Aliquots of each suspension were run in the Warburg for 140 minutes, half of these in each case being run with 0.5 ml. of 0.1 M. glucose as substrate, and the other half without substrate to determine the endogenous respiration. The respiration in each case was calculated as microliters of oxygen per milligram dry weight of cell suspension. The results obtained are shown in Table 7.

These results show that a slight increase in respiration per milligram of dry weight of the suspension was obtained with the most concentrated cell suspension used. This increase was obtained with the endogenous respiration as well as with the respiration in the presence of glucose. Although errors of measurement may be less in the case of the highest cell concentration, since larger weights and volumes are involved, the differences obtained among the three concentrations were not great. Whenever possible in the subsequent experiments a cell concentration, of 20 per cent transmission, or greater, was used, the 20 per cent transmission suspension in most cases being the most convenient one.

TABLE 7

RESPIRATION MEASUREMENTS ON CELLS USING DIFFERENT SUSPENSION* CONCENTRATIONS

Suspension Transmission	Oxygen absorbed/mg. of cells/ 1h0 min. Endogenous	Substrate**	Substrate minus Endogenous Ave.	Dry Weight
per cent	uL	uL	uL	mg/ml
10	18.h	28.8		
	19.6	28.5	9.7	5.68
20	11.9	21.0		
	13.1	20.2	8.1	2.79
35	1h.9	22.0		
	11.8	----***	8.6	1.37

* Suspension prepared from h8 hour peptone cells.
** Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.
***Sample discarded because of a leak in the connections.

6. Effect of storage of the cell suspension at 10° C., on respiration measurements. The effect of cold storage was investigated to determine its effect on subsequent respiration measurements, since experiments could be accomplished much more efficiently if many variables could be studied on one suspension. This would, of course, require that the suspension be stable during such storage. Accordingly, a washed suspension of h8 hour cells grown on the basal medium with 0.172 per cent added peptone as the nitrogen source was prepared. The suspension was diluted with pH 6.0 buffer to 20 per cent transmission. Some of the suspension was used in the Warburg flasks immediately after it had been prepared. The rest of the suspension was stored in a cork stoppered

125 ml. Erlenmeyer flask in the refrigerator at 10° C., for 24 hours before it was used. A 5 ml. aliquot of the original suspension was used to determine the dry weight of the cell suspension. This dry weight value was assumed to be constant during storage. Eight replicate flasks were set up for both the suspension and the stored portion of the suspension and were run in the Warburg for 140 minutes. Half of the flasks in each case were run with 0.5 ml. of 0.1 M glucose as substrate, and the other half of the flasks were run without substrate to determine the endogenous respiration. The respiration was claculated as microliters of oxygen per milligram dry weight of the suspension. The results from both the original suspension and that after 24 hours storage are reported in Table 8.

TABLE 8

RESPIRATION MEASUREMENTS ON A CELL SUSPENSION*
BEFORE AND AFTER 24 HOURS COLD STORAGE

Suspension	Oxygen absorbed/ mg. of cells/ 140 minutes		
	Endogenous	Substrate**	Substrate minus endogenous ave.
	uL	uL	uL
Original	58.1	93.4	
	56.5	86.1	
	55.1	95.6	
	56.8	94.8	
Ave.	56.6	92.5	35.9
After 24 hours	65.5	86.7	
	56.4	73.9	
	66.3	83.0	
	62.2	76.3	
Ave.	62.6	80.0	17.4

* Suspension prepared from 48 hour peptone cells and diluted to 20 per cent transmission. Dry weight was 1.68 mg. per ml. of suspension.
**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.

The results show that in the stored suspension the endogenous respiration increased slightly, whereas the glucose oxidative ability decreased. A decrease in both would be expected if the only effect of cold storage were a decrease in activity of the cells. The apparent slight decrease in values for substrate minus endogenous respiration shown in Table 4 after the storage for one or two hours at 10° C., may result from the storage rather than from an effect of shaking rate. In view of the change which occurs, it was thought inadvisable to store any suspensions before they were run in the Warburg. Consequently, the respiration of all cell suspensions was measured as soon as the suspensions were prepared.

7. Effect of concentration of glucose on respiration measurements. Although previous experiments with glucose showed no decrease in the rate of oxygen uptake during the time intervals of study, it was thought advisable to determine whether the concentration of glucose used had any effect on the respiration measurements. Accordingly, a washed suspension of 48 hour cells grown on the basal medium with 0.17? per cent added peptone was prepared to study the effect of the concentration of glucose added as substrate to the Warburg flasks. The suspension was diluted with pH 6.0 buffer to a final turbidity of 25 per cent transmission. A 10 ml. aliquot was used for the determination of the dry weight of the suspension. The following amounts- 0.4, 0.5, 0.6, and 0.7 ml. - of 0.1 M. glucose were added to duplicate flasks. All of the samples were run in the Warburg for 140 minutes. The respiration was calculated as microliters of oxygen per milligram dry weight of the suspension. The results are given in Table 9.

TABLE 9

RESPIRATION MEASUREMENTS ON A CELL SUSPENSION* WITH DIFFERENT CONCENTRATIONS OF GLUCOSE ADDED AS SUBSTRATE

0.1 M. Glucose	Oxygen absorbed/mg. of cells/1½ min.
ml.	uL
0.0	21.8
	20.6
0.4	30.7
	30.7
0.5	28.7
	28.6
0.6	31.0
	30.9
0.7	35.2
	36.7

*Suspension prepared from 48 hour peptone cells and diluted to 25 per cent transmission. Dry weight was 2.06 mg. per ml. of suspension.

The results in Table 9 show that 0.4 ml. of 0.1 M. glucose is sufficient substrate so that it is not a limiting factor in the respiration measurements. However, to be sure that an excess of glucose was present 0.5 ml. of 0.1 M glucose was chosen as the amount to be used in further experiments in which this substrate was used.

8. A study of cells grown on the basal medium with different nitrogen sources. Early work by Stephens showed that a medium containing only L-glutamic acid and L-arginine monohydrochloride as the source of nitrogen gave very good growth of A. gossypii; however, only small amounts of riboflavin were formed. When L-leucine or L-leucine and

L-histidine were added to this medium, A. gossypii produced noticeable quantities of riboflavin, even though the growth was somewhat decreased. It was thought to be of interest to study the respiration of cells grown on each of these media, both with and without glucose added as a substrate, using cells grown on peptone medium as a control.

Four different media were prepared, with different amounts and sources of nitrogen, for growing cells of different ages for respiration measurements. The media were prepared using the basal medium with the amounts and sources of nitrogen added as follows: 1- peptone (P) medium, peptone to make a concentration of 0.172 per cent; 2- glutamic acid-arginine (GA) medium, L-glutamic acid and L-arginine monohydrochloride to give concentrations of 0.21 and 0.012 per cent respectively; 3- glutamic acid-arginine-leucine (GAL) medium, L-glutamic acid, L-arginine monohydrochloride and L-leucine to give concentrations of 0.21, 0.012 and 0.25 per cent respectively; and 4- glutamic acid-arginine-leucine-histidine (GALH) medium, L-glutamic acid, L-arginine monohydrochloride, L-leucine and L-histidine to give concentrations of 0.21, 0.012, 0.25 and 0.018 per cent respectively. Four flasks of each medium were inoculated to obtain 24 hour cells, whereas two flasks of each medium were inoculated to obtain each of the 48, 72 and 96 hour cells. The same inoculum was used for all flasks. After incubation the contents of each flask were diluted with distilled water to the original volume (20 ml.) and pH and turbidity measurements were made. An examination of these cultures with ultraviolet light was made in order to determine the first appearance of riboflavin by fluorescence in each medium. To compare some of the data more advantageously, optical density values were

calculated from the average per cent transmission values. The results are given in Table 10. Curves of optical density values are shown in Figure I.

The above cells were then washed in the usual manner and diluted with pH 6.0 buffer to prepare cell suspensions with 40 per cent transmission. A 10 ml. aliquot of each suspension was filtered, dried at 78° C., and weighed to determine the dry weight of each suspension. Duplicate samples from the cell suspensions for each medium and each age of cells were run in the Warburg for 140 minutes without substrate to determine the endogenous respiration. Duplicate samples were also run as above with 0.5 ml. of 0.1 M. glucose as substrate in each flask. The oxygen uptake in each case was calculated as microliters of oxygen per milligram dry weight of cell suspension in the Warburg flask. The results obtained are given in Table 11. A curve of average endogenous respiration values from Table 11 is shown in Figure II.

In order to determine the rate of growth on the basis of dry weight of the cells another set of four media was prepared, inoculated and incubated in the same manner. After 24, 48, 72 and 96 hours incubation the contents of each flask were diluted with distilled water to the original volume (20 ml.) and pH and turbidity measurements were made on each culture medium. Then the dry weight of cells in the 20 ml. volume of culture medium in each case was determined by combining the contents of the flasks for each age of cells for each media, filtering a 10 ml. aliquot of this suspension, drying it at 78° C., and weighing it. Optical density values were calculated from average per cent transmission values. These results are given in Table 12. Optical density values at different

ages, given in Table 12, are plotted in Figure III. Curves are shown in Figure IV for the average dry weight values, at different ages, presented in Table 12.

An examination of the data from Tables 10 and 12 shows that there is an inhibition or delay of growth of A. gossypii on the GA, GAL and GALH media compared with that on the P medium. Figure IV shows that the P medium gives a maximal weight of cells at 48 hours. The other media do not show a maximum because they apparently have not yet reached that point. This is indicated by the large increases in dry weights between 72 and 96 hours for these media. The decrease in weight of cells from the P medium beyond 48 hours is probably due to changes in the older cells with a loss of their contents to the solution, a common occurrence in old cells. This decrease is not reflected in the turbidity measurements possibly because the older cells are still exhibiting the same optical properties as the younger cells, even though substances have passed out of the cells in the former case.

A comparison of the turbidity values in Tables 10 and 12 and in Figures I and III indicates that growth occurred earlier in each of the media the first time the cultures were prepared. In other words, there is a consistent delay in growth shown by all of the cultures that were prepared the second time. This may have been due to differences in the inocula either as to amount of cells or to amount of the culture medium present, since, as has been mentioned above, it is difficult to prepare homogeneous dispersions of the organism because it grows in clumps. Although there is this delay in growth the second time cultures were made, the same comparative results were obtained as with the first cultures.

When leucine is added to GA medium the rate of growth is delayed over that with GA medium alone up to 72 hours. At 96 hours, however, the growth in the GAL medium has surpassed that in the GA medium.

TABLE 10

TURBIDITY, pH, AND RIBOFLAVIN MEASUREMENTS ON 24, 48, 72 AND 96 HOUR CULTURES GROWN ON BASAL MEDIA WITH DIFFERENT NITROGEN SOURCES

Age		Transmission (T)	Optical Density	pH	Riboflavin*
hrs.		per cent	2-log % T		
			Peptone Medium		
24		36.0		6.30	
		38.0		6.30	
		35.0		6.30	
		34.5		6.35	
	Ave.	35.9	0.444	6.31	+
48		18.0		6.05	
		18.0		6.05	
	Ave.	18.0	0.745	6.05	+
72		12.5		6.05	
		12.0		5.80	
	Ave.	12.3	0.910	5.93	+
96		10.0		5.75	
		10.5		5.55	
	Ave.	10.3	0.987	5.65	+
			Glutamic Acid-Arginine Medium		
24		49.0		6.50	
		48.5		6.45	
		41.5		6.55	
		55.0		6.40	
	Ave.	48.5	0.314	6.48	-

(Table continued on following page)

TABLE 10 (continued)

Age		Transmission (T)	Optical Density	pH	Riboflavin*
hrs.		per cent	2-log % T		
		Glutamic Acid-Arginine Medium (continued)			
48		17.5		6.85	
		15.0		6.95	
	Ave.	16.3	0.789	6.90	-
72		10.5		6.55	
		11.5		6.75	
	Ave.	11.0	0.959	6.65	-
96		9.5		6.80	
		9.5		6.75	
	Ave.	9.5	1.022	6.78	-
		Glutamic Acid-Arginine-Leucine Medium			
24		87.5		6.45	
		89.5		6.45	
		88.5		6.45	
		88.5		6.45	
	Ave.	88.5	0.033	6.45	-
48		25.0		6.70	
		19.0		6.75	
	Ave.	22.0	0.658	6.73	+
72		10.5		6.85	
		16.0		6.86	
	Ave.	13.3	0.878	6.85	+
96		9.0		6.75	
		9.5		7.05	
	Ave.	9.3	1.034	6.90	+
		Glutamic Acid-Arginine-Leucine-Histidine Medium			
24		90.0		6.50	
		90.0		6.55	
		91.5		6.55	
		91.5		6.50	
	Ave.	90.8	0.042	6.53	-

(Table continued on following page)

TABLE 10 (continued)

Age	Transmission (T)	Optical Density	pH	Riboflavin*
hrs.	per cent	2-log % T		
Glutamic Acid-Arginine-Leucine-Histidine Medium (continued)				
48	68.0		6.40	
	70.5		6.40	
Ave.	69.3	0.160	6.40	+
72	19.0		6.65	
	17.0		6.70	
Ave.	18.0	0.745	6.68	+
96	14.5		6.95	
	10.5		7.45	
Ave.	12.5	0.903	7.20	+

*(+) indicates fluorescence. (-) indicates no fluorescence.

This might be considered as a result of the extra amount of nitrogen present from the added leucine. If such were the case, however, this increase might be expected to occur to a greater extent when the medium contained even more nitrogen as in the GALH medium. This did not occur. The maximum growth as determined by the dry weight in the GA medium is 74 mg. If nitrogen in the dry weight of yeasts is assumed to average about 10 per cent, 74 mg. of dried cells would contain approximately 7.4 mg. of nitrogen. The total amount of nitrogen available from the amounts of glutamic acid and arginine used is 23.15 mg. Thus nitrogen is not considered to be a limiting factor.

FIGURE I

A COMPARISON OF OPTICAL DENSITY VALUES FROM 24, 48, 72 AND 96 HOUR CULTURES GROWN ON BASAL MEDIA WITH DIFFERENT NITROGEN SOURCES FOR SUSPENSIONS FOR RESPIRATION MEASUREMENTS

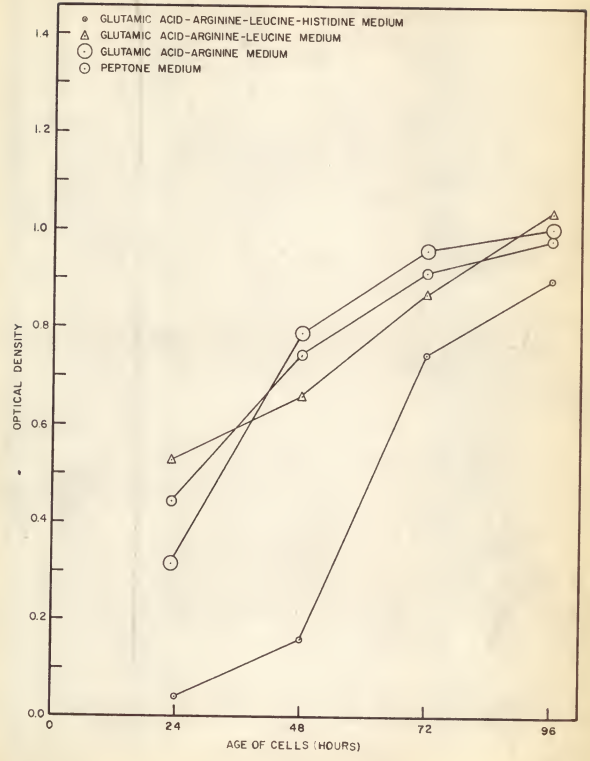

GLUTAMIC ACID-ARGININE-LEUCINE-HISTIDINE MEDIUM
GLUTAMIC ACID-ARGININE-LEUCINE MEDIUM
GLUTAMIC ACID-ARGININE MEDIUM
PEPTONE MEDIUM
OPTICAL DENSITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
AGE OF CELLS (HOURS)

TABLE 11

RESPIRATION MEASUREMENTS ON SUSPENSIONS* OF 24, 48, 72 AND 96 HOUR CELLS GROWN ON BASAL MEDIA WITH DIFFERENT NITROGEN SOURCES

Age	Dry Weight	Oxygen absorbed/mg. of cells/140 min. Endogenous	Substrate**	Substrate minus Endogenous Ave.
hrs.	mg./ml.	uL	uL	uL
		Peptone Medium		
24	0.77	52.8	107.5	
		58.4	91.8	
		Ave. 55.6	99.7	44.1
48	1.18	65.8	77.5	
		52.9	67.5	
		Ave. 59.4	72.5	13.1
72	0.99	43.4	52.6	
		39.8	56.1	
		Ave. 41.6	54.4	12.8
96	1.39	40.5	54.1	
		36.0	---***	
		Ave. 38.3	54.1	15.8
		Glutamic Acid-Arginine Medium		
24	0.85	56.7	90.3	
		48.8	110.0	
		Ave. 52.8	100.2	47.4
48	1.26	50.4	72.3	
		48.2	72.4	
		Ave. 49.3	72.4	23.1
72	1.16	28.0	44.8	
		33.9	50.8	
		Ave. 31.0	47.8	16.8

(Table continued on following page)

TABLE 11 (continued)

Age	Dry Weight		Oxygen absorbed/mg. of cells/1h0 min. Endogenous	Substrate**	Substrate minus Endogenous Ave.
hrs.	mg./ml.		uL	uL	uL
		Glutamic Acid-Arginine Medium (continued)			
96	1.37		29.4	28.9	
			24.7	38.1	
		Ave.	27.1	33.5	6.4
		Glutamic Acid-Arginine-Leucine Medium			
24	0.99		84.5	157.9	
			93.1	153.7	
		Ave.	88.8	155.8	67.0
48	1.31		45.9	84.4	
			68.5	92.1	
		Ave.	57.2	88.3	31.1
72	0.88		32.2	43.3	
			26.1	45.0	
		Ave.	29.2	44.2	15.0
96	1.73		37.0	44.7	
			35.8	50.5	
		Ave.	36.4	42.6	6.2
		Glutamic Acid-Arginine-Leucine-Histidine Medium			
24	0.60		56.1	91.2	
			---****	86.5	
		Ave.	56.1	88.8	32.7
48	2.47		116.9	168.3	
			-----****	170.4	
		Ave.	116.9	169.4	52.5
72	1.12		60.0	71.2	
			---****	90.5	
		Ave.	60.0	80.9	20.9

(Table continued on following page)

TABLE 11 (continued)

Age	Dry Weight	Oxygen absorbed/mg. of cells/1½0 min. Endogenous	Substrate**	Substrate minus Endogenous Ave.
hrs.	mg./ml.	uL	uL	uL
	Glutamic Acid-Arginine-Leucine-Histidine Medium (continued)			
96	1.36	56.9	67.9	
		---***	48.2	
	Ave.	56.9	58.1	1.2

*Suspensions were diluted to 40 per cent transmission.
**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.
***Sample discarded because of a leak in the connections.
****Duplicate samples omitted because of Warburg space limitations.

The data from Table 11 and Figure II show that the oxygen uptake values are unusually high at 24 hours in the case of the GAL medium and at 48 hours in the case of the GALH medium. The data show that this increase results from an increase in the endogenous respiration. The GA and P media do not show this large increase in oxygen uptake. It may be that it occurs with these media at ages not measured. The fact that the stimulation of respiration occurs along with inhibition of growth may indicate that non-growth pathways, such as those leading to the formation of riboflavin, which use substances needed for growth, are favored, or that there is a time lag during which the synthesis of a substance or substances required for growth occurs.

The early occurrence of riboflavin in the P medium may be due to the supply of readily available precursor materials present. The synthesis of these precursor materials may be responsible for the peaks in

FIGURE II

A COMPARISON OF ENDOGENOUS RESPIRATION MEASUREMENTS ON SUSPENSIONS OF 24, 48, 72 AND 96 HOUR CELLS GROWN ON BASAL MEDIA WITH DIFFERENT NITROGEN SOURCES.

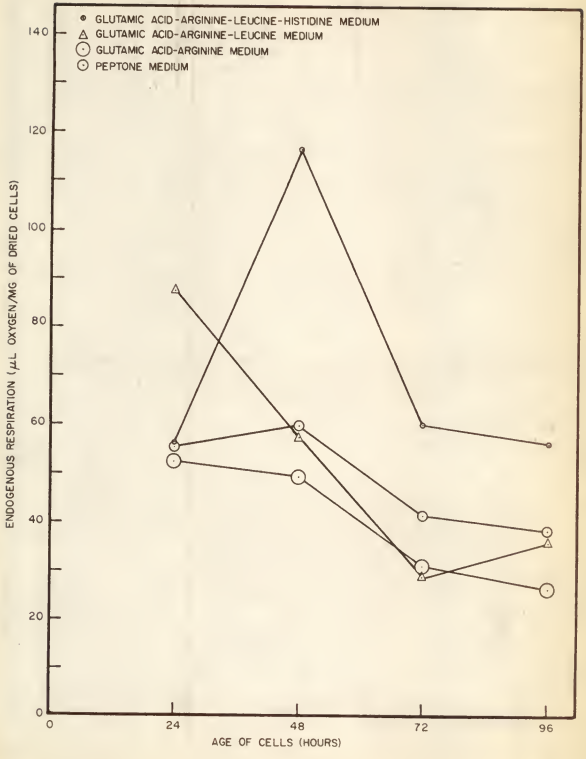

GLUTAMIC ACID-ARGININE-LEUCINE-HISTIDINE MEDIUM
GLUTAMIC ACID-ARGININE-LEUCINE MEDIUM
GLUTAMIC ACID-ARGININE MEDIUM
PEPTONE MEDIUM
ENDOGENOUS RESPIRATION (μL OXYGEN/MG OF DRIED CELLS)
0
20
40
60
80
100
120
140
0
24
48
72
96
AGE OF CELLS (HOURS)

TABLE 12

TURBIDITY, pH, AND DRY WEIGHT MEASUREMENTS ON 24, 48, 72 AND 96 HOUR CULTURES GROWN ON BASAL MEDIA WITH DIFFERENT NITROGEN SOURCES

Age		Transmission (T)	Optical Density	pH	Dry Weight
hrs.		per cent	2-log % T		mg./20 ml.
		Peptone Medium			
24		82.0		6.00	
		80.0		5.90	
		80.0		5.85	
		79.5		6.05	
	Ave.	80.4	0.094	5.95	21.4
48		9.5		5.50	
		8.0		5.20	
	Ave.	8.8	1.059	5.35	135.6
72		6.0		6.80	
		6.5		6.85	
	Ave.	6.3	1.204	6.83	123.2
96		5.0		7.55	
		6.0		7.75	
	Ave.	5.5	1.260	7.65	113.4
		Glutamic Acid-Arginine Medium			
24		97.0		6.40	
		97.0		6.45	
		96.5		6.50	
		97.0		6.50	
	Ave.	96.9	0.013	6.46	6.1
48		95.0		6.40	
		84.0		6.50	
	Ave.	89.5	0.048	6.45	8.2
72		32.0		6.70	
		27.0		6.25	
	Ave.	29.5	0.530	6.48	30.2

(Table continued on following page)

TABLE 12 (continued)

Age		Transmission (T)	Optical Density	pH	Dry Weight
hrs.		per cent	2-log % T		mg./20 ml.
		Glutamic Acid-Arginine Medium (continued)			
96		16.0		6.95	
		14.0		6.80	
	Ave.	15.0	0.824	6.88	74.0
		Glutamic Acid-Arginine-Leucine Medium			
24		97.5		6.35	
		98.0		6.35	
		99.0		6.35	
		98.0		6.40	
	Ave.	98.1	0.009	6.36	8.6
48		91.0		6.35	
		86.0		6.40	
	Ave.	88.5	0.053	6.38	8.0
72		51.0		6.35	
		46.0		6.40	
	Ave.	48.0	0.319	6.38	11.2
96		12.0		6.90	
		9.0		6.85	
	Ave.	10.5	0.979	6.88	98.8
		Glutamic Acid-Arginine-Leucine-Histidine Medium			
24		96.0		6.40	
		97.0		6.40	
		96.0		6.40	
		96.0		6.40	
	Ave.	96.2	0.017	6.40	6.2
48		94.0		6.45	
		90.0		6.45	
	Ave.	92.0	0.036	6.45	5.8
72		92.0		6.25	
		89.0		6.25	
	Ave.	90.5	0.043	6.25	5.8

(Table continued on following page)

TABLE 12 (continued)

Age	Transmission (T)	Optical Density	pH	Dry Weight
hrs.	per cent	2-log % T		mg./20 ml.
Glutamic Acid-Arginine-Leucine-Histidine Medium (continued)				
96	20.5		6.50	
	16.5		6.65	
Ave.	18.5	0.733	6.58	63.2

respiration values obtained at 24 hours and at 48 hours with the GAL and the GALH media respectively. It is interesting to note that the riboflavin formation, except that in the P medium, occurs in the two media giving the greatest delay in growth. It has been found independently by Dikanshaya, Nickelson and Stephens that most of the riboflavin usually forms after a period of rapid growth when the organisms are growing only slowly. Thus it would seem that non-growth pathways leading to the formation of riboflavin may be favored whenever little growth is occurring, whether such slow growth is after a period of rapid growth or is due to inhibition or delay caused by certain substances.

9. Further studies on techniques to obtain more consistent measurements of respiration. Because of variations noted on respiration measurements on duplicate aliquots of a cell suspension further attempts were made to obtain better agreement between duplicate samples. Since the data in Experiment 1 showed that more consistent, although somewhat lower, results were obtained between duplicate samples of a suspension homogenized for 10 seconds, it was felt that this effect should be investigated further. Glass beads were added to the suspension so that it

FIGURE III

A COMPARISON OF OPTICAL DENISTY VALUES FROM 24, 48, 72 AND 96 HOUR CULTURES GROWN ON BASAL MEDIA WITH DIFFERENT NITROGEN SOURCES FOR GROWTH MEASUREMENTS

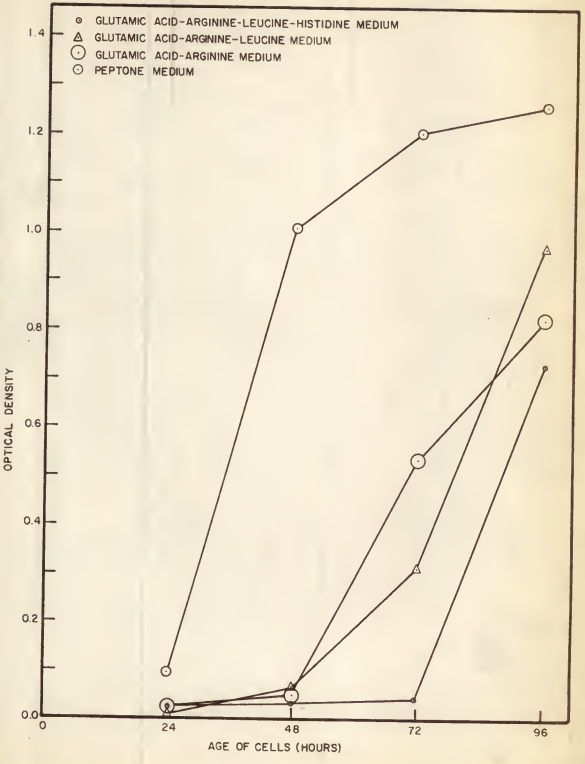
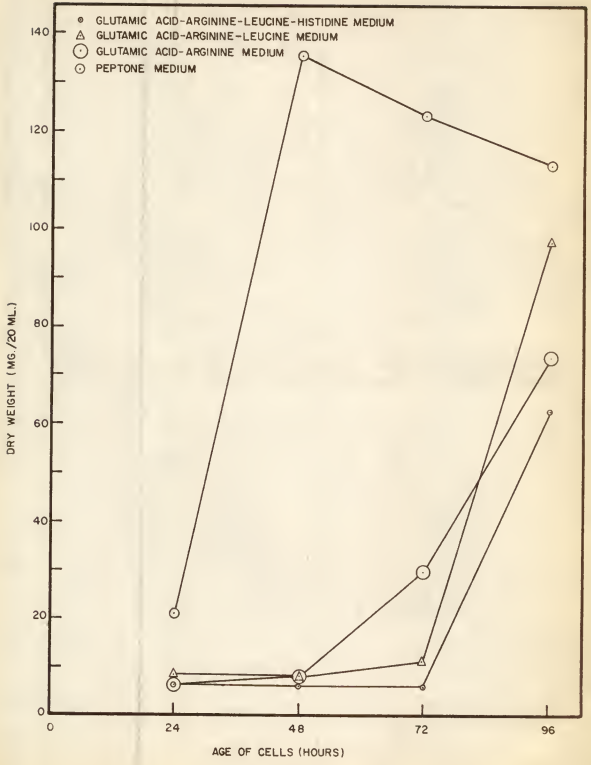

GLUTAMIC ACID-ARGININE-LEUCINE-HISTIDINE MEDIUM
GLUTAMIC ACID-ARGININE-LEUCINE MEDIUM
GLUTAMIC ACID-ARGININE MEDIUM
PEPTONE MEDIUM
OPTICAL DENSITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
AGE OF CELLS (HOURS)

FIGURE IV

A COMPARISON OF DRY WEIGHT MEASUREMENTS ON 24, 48, 72 AND 96 HOUR CULTURES GROWN ON BASAL MEDIA WITH DIFFERENT NITROGEN SOURCES.


GLUTAMIC ACID-ARGININE-LEUCINE-HISTIDINE MEDIUM
GLUTAMIC ACID-ARGININE-LEUCINE MEDIUM
GLUTAMIC ACID-ARGININE MEDIUM
PEPTONE MEDIUM
DRY WEIGHT (MG./20 ML.)
0
20
40
60
80
100
120
140
0
24
48
72
96
AGE OF CELLS (HOURS)

could be swirled with the beads between each removal of cells for the Warburg, thereby keeping the suspension more homogeneous.

Accordingly, a suspension of 24 hour cells grown on the basal medium with 0.172 per cent added peptone was prepared to study the effect of a 10 second homogenization period with the addition of glass beads to the final suspension. The suspension was homogenized in the Waring blendor for 10 seconds and diluted with pH 6.0 buffer to 40 per cent transmission. It was placed in a 125 ml. Erlenmeyer flask which contained enough glass beads to cover the bottom of the flask. The suspension was swirled 3 or 4 times with the beads before each 1 ml. removal of cells for the Warburg flasks. A 7 ml. aliquot of this suspension was filtered, dried at 78° C., and weighed to determine the dry weight. Duplicate aliquots of the suspension were run with 0.5 ml. of 0.1 M. glucose as substrate and with no substrate to determine the endogenous respiration. All samples were run for 140 minutes in the Warburg and the respiration was calculated as microliters of oxygen per milligram of dried cell suspension. The results obtained are given in Table 13.

The data from Table 13 show that somewhat better duplication of results is obtained by the above changes in technique.

In order to study the effect of the addition of glass beads to cell suspensions homogenized for different periods of time, a suspension of 48 hour cells grown on the peptone medium with 0.172 per cent added peptone was prepared. This suspension was placed in the Waring blendor and homogenized. Aliquots were removed from the blendor after the following times of homogenization: 5, 10, 15, 20, 25, 30, 60 and 120 seconds. Each aliquot was diluted with pH 6.0 buffer to form a suspension of

TABLE 13

RESPIRATION MEASUREMENTS ON ALIQUOTS OF A 24 HOUR CELL SUSPENSION* SHAKEN WITH GLASS BEADS

Oxygen absorbed/ mg. of cells/ 1h0 min.		
Endogenous	Substrate**	Substrate minus Endogenous
uL	uL	uL
37.8	74.3	36.5
38.0	79.2	41.2
44.5	73.1	28.6
39.3	68.2	28.9

*Suspension was prepared from cells grown on peptone medium and diluted to 40 per cent transmission.

**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.

20 per cent transmission. Each of these suspensions was then transferred to a 125 ml. Erlenmeyer flask which contained enough glass beads to completely cover the bottom of the flask. A 10 ml. aliquot from the 5 second homogenized suspension and a 5 ml. aliquot from each of the other suspensions were filtered, dried at 78° C., and weighed to determine the dry weight of each suspension. Each suspension was swirled with the glass beads before each 1 ml. removal of cells for the Warburg flasks. To each Warburg flask was added 0.5 ml. of 0.1 M. glucose as substrate. All samples were run in the Warburg for 90 minutes to determine the endogenous respiration. At the end of this time the substrate was tipped in and the samples were run for an additional 90 minutes to determine the respiration in the presence of glucose. The respiration in each case was calculated as microliters of oxygen per milligram of dried suspension. The results are given in Table 14.

TABLE 14

RESPIRATION MEASUREMENTS ON A 48 HOUR PEPTONE CELL SUSPENSION* AFTER DIFFERENT PERIODS OF HOMOGENIZATION AND SHAKING WITH GLASS BEADS

Time of homogenization	Dry weight	Oxygen uptake/mg. of cells/140 min. Endogenous	Substrate**	Substrate minus twice endogenous
				Ave.
sec.	mg/ml	uL	uL	uL
5	2.41	33.0	75.1	
		31.9	71.8	
	Ave.	32.5	73.9	8.9
10	3.00	32.1	69.7	
		30.1	61.2	
	Ave.	31.1	65.5	3.3
15	2.65	22.4	52.7	
		28.0	60.8	
	Ave.	25.2	56.8	6.4
20	2.50	32.3	67.8	
		30.7	67.2	
	Ave.	31.5	67.5	4.5
25	3.20	23.1	52.9	
		24.2	54.0	
	Ave.	23.6	53.4	6.2
30	2.50	18.4	41.5	
		17.3	48.5	
	Ave.	17.9	45.0	9.2
60	7.00	7.2	16.5	
		11.4	24.2	
	Ave.	9.5	20.3	1.3
120	5.00	6.8	12.5	
		6.1	13.5	
	Ave.	6.5	13.0	0.0

*Suspensions were diluted to 20 per cent transmission.
**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.

These results show that the 5 second homogenization gives higher values for glucose and slightly higher values for endogenous respiration than do the 10 second and longer homogenizations. The results are similar to those obtained in Experiment 1; that is, there is a gradual decrease in respiration values as the length of time of homogenization is increased. Although there is a slight decrease in values for the 10 second homogenization, it was beleived that this homogenization time, with the addition of glass beads to the suspensions later, would help provide more uniform sampling. Thus, the succeeding experiments were handled in the above manner, except as otherwise noted.

10. A study of cells grown on the basal medium with glutamic acid, arginine and glycine as the nitrogen sources. Stephens has shown that A. gossypii grown on a medium containing only arginine and glutamic acid as the nitrogen sources gives very good growth but only slight riboflavin production. The addition of glycine to this medium, however, caused a large increase in the riboflavin production with a slight decrease in growth. To study the effect of addition of glycine on the respiration measurements a medium was prepared which contained L-glutamic acid, L-arginine monohydrochloride and glycine in concentrations of 0.21, 0.012 and 0.018 per cent respectively. This medium was inoculated in the usual manner and incubated for 48 hours before cell suspensions were prepared. The cell suspension was diluted with pH 6.0 buffer to 20 per cent transmission. A 10 ml. aliquot was used for the determination of dry weight of the cell suspension. Quadruplicate aliquots from the suspension were run in the Warburg for 140 minutes both without substrate to determine the endogenous respiration and with 0.5 ml. of 0.1 M. glucose as

substrate in each flask. Respiration in each case was calculated as microliters of oxygen per milligram of dried cell suspension. The results obtained are shown in Table 15. Examination of the culture under ultraviolet light showed the presence of riboflavin by fluorescence.

TABLE 15

RESPIRATION MEASUREMENTS ON A SUSPENSION* PREPARED FROM CELLS GROWN ON THE BASAL MEDIUM WITH GLUTAMIC ACID, ARGININE AND GLYCINE ADDED AS THE NITROGEN SOURCES

	Oxygen absorbed/ mg. of cells/ 1h0 min.		
	Endogenous	Substrate**	Substrate minus Endogenous Ave.
	uL	uL	uL
	30.9	41.0	
	28.0	39.6	
	33.3	42.9	
	33.7	43.0	
Ave.	32.0	41.6	9.6

*Suspension was prepared from 48 hour cells and diluted to 20 per cent transmission.

**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.

A comparison of the date from Table 15 and from Table 11 in Experiment 8 shows that the respiration measurements for both endogenous and substrate on glutamic acid, arginine and glycine grown cells are lower than those previously obtained with peptone, glutamic acid-arginine, glutamic acid-arginine-leucine and glutamic acid-arginine-leucine-histidine media. This is in spite of the fact that the amount of riboflavin produced with the glycine medium is greater than that produced in each

of the other cases except that of peptone. As pointed out in Experiment 8, however, the respiration maximum may be at an age other than 48 hours.

Since copper and iron are involved in many oxidation-reduction enzyme systems it was felt that it might be of interest to add small amounts of these metals to a medium containing glycine as well as glutamic acid and arginine to determine whether they had any effect on respiration measurements. Accordingly, a medium was prepared as above except that 1.0 mg. of $FeSO_4$ and 0.5 mg. of $CuSO_4$ were added. The medium was inoculated and after 48 hours incubation a cell suspension was prepared and diluted with pH 6.0 buffer to 20 per cent transmission. A ten ml. aliquot was used for the determination of dry weight of the suspension. Triplicate aliquots of this suspension were run in the Warburg for 140 minutes without substrate to determine the endogenous respiration and with 0.5 ml. of 0.1 M. glucose as substrate. The results are shown in Table 16.

A comparison of the data in Tables 15 and 16 shows that the addition of copper and iron gives about a 20 per cent increase in the endogenous respiration and about a 50 per cent increase in the respiration in the presence of glucose. These increases may be due to the increased activity of copper and/or iron containing enzymes. The increased values for substrate minus endogenous respiration may also result from increased activity of copper and/or iron containing enzymes, assuming that the rate of oxygen absorption without substrate is limited by the amount of oxidizable substances in the cells. That is, the copper and/or iron containing enzymes may be in excess when the cells are grown on the medium containing the copper and iron. Thus, when substrate is

TABLE 16

RESPIRATION MEASUREMENTS ON A SUSPENSION* PREPARED FROM CELLS GROWN ON THE BASAL MEDIUM WITH GLUTAMIC ACID, ARGININE AND GLYCINE ADDED AS THE NITROGEN SOURCES AND WITH ADDED COPPER AND IRON

	Oxygen absorbed/ mg. of cells/ 1h0 min.		
	Endogenous	Substrate**	Ave. Substrate minus Endogenous
	uL	uL	uL
	h1.8	62.2	
	38.0	65.h	
	h1.6	62.7	
Ave.	h0.5	63.h	22.9

*Suspension was prepared from h8 hour cells and diluted to 20 per cent transmission. Dry weight was 2.h6 mg. per ml. of suspension.
**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.

added, there is a larger increase in oxygen uptake over endogenous respiration values.

11. A study of cells grown on peptone, yeast extract and glucose medium. The medium used for maintenance of the culture, which was previously described, when prepared as a liquid medium without the agar was found to give excellent growth and riboflavin production. Therefore, it was thought advisable to have some information on the respiration obtained using this medium to serve as a sort of control or optimal medium for the growth and production of riboflavin by A. gossypii. Accordingly, a medium containing 2.0 per cent glucose, 1.0 per cent peptone and 0.5 per cent yeast extract was prepared. The medium was inoculated and after 2h and h8 hours incubation cell suspensions were prepared from these cultures. Each cell suspension was diluted with pH 6.0 buffer to ten

per cent transmission. A 10 ml. aliquot from each suspension was used to determine the dry weight. For each suspension duplicate aliquots were run in the Warburg without substrate to determine the endogenous respiration and duplicate aliquots were run with 0.5 ml. of 0.1 M. glucose as substrate, all respiration measurements covering a 1h0 minute period. The respiration in each case was calculated as microliters of oxygen absorbed per milligram of dried cell suspension. The results are given in Table 17.

TABLE 17

RESPIRATION MEASUREMENTS ON A CELL SUSPENSION* PREPARED FROM CELLS GROWN ON A MEDIUM CONTAINING PEPTONE, YEAST EXTRACT AND GLUCOSE

Age	Dry weight		Oxygen absorbed/ mg. of cells/ 1h0 min. Endogenous	Substrate**	Substrate minus Endogenous Ave.
hrs.	mg/ml		uL	uL	uL
2h	5.68		h4.7	91.0	
			36.6	90.0	
		Ave.	h0.7	90.5	h9.8
h8	h.62		27.7	70.8	
			28.0	78.h	
		Ave.	27.9	7h.6	h6.7

*Cell suspensions were diluted to 10 per cent transmission.
**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.

A comparison of the data from Tables 11 and 17 shows that the suspension from the peptone and yeast extract grown cells at 2h hours has a much lower endogenous respiration and a slightly lower respiration

in the presence of glucose than the suspension of cells from the basal medium with peptone. At 48 hours the peptone and yeast extract grown cells have only about one-half the endogenous respiration shown by the cells grown on peptone medium but when glucose is added both cell suspensions show about the same respiration.

These results might be explained by assuming that the cells in this experiment at 24 hours and 48 hours age are further along in their growth phases. Since the medium used in this experiment allows more rapid growth, it might be expected that the cells grown on this medium might be further ahead in the growth phase than those grown on the peptone medium. As has been said earlier, respiration values are normally higher in rapidly growing cells. These values become smaller as the cells go into the stationary phase of growth. The more rapid growth on the peptone and yeast extract medium probably is due to the presence of B vitamins in the yeast extract.

When respiration values of the cells in this experiment are compared with those for cells grown on media where inhibition or delay of growth occurs such as in the GAL or GALH media, further emphasis is placed on the interpretation of respiration values in relation to growth phases. One exception to this has already been noted. That is the unusually high respiration values that occurred with cells from the above two media that inhibited or delayed growth, which were assumed to be related to riboflavin formation or the formation of substances needed for growth.

12. Respiration measurements at pH 6.0 on cell suspensions using amino acids as substrate. Since amino acids seem to be involved in the production of riboflavin by A. gossypii, a study of respiration, using amino acids as substrates, was made of cells grown on a medium where no riboflavin is produced and on media where noticable amounts of riboflavin are produced. Three media were prepared using the basal medium with the following concentrations of peptone or amino acids: 1- peptone medium- 0.172 per cent peptone; 2- glutamic acid-arginine medium- 0.21 per cent L-glutamic acid and 0.012 per cent L-arginine monohydrochloride; 3- glutamic acid-arginine-glycine medium- 0.21 per cent L-glutamic acid, 0.012 per cent L-arginine monohydrochloride and 0.018 per cent glycine.

Fifty milligrams of each of the following amino acids was dissolved in sufficient pH 6.0 buffer-water mixture to make 100 ml. of each solution of amino acid: glycine, L-cystine, L-leucine, DL-threonine, DL-alpha-alanine, L-lysine monohydrochloride, L-proline, L-tyrosine, DL-tryptophan, DL-phenylalanine, DL-isoleucine, DL-serine, L-glutamic acid, L-arginine monohydrochloride and L-asparagine (a derivative of aspartic acid). Since all of the tyrosine did not dissolve, it was used as a saturated solution. The pH of each of the above solutions, except glutamic acid, was between pH 6.0 and pH 6.4. The pH of the glutamic acid solution was adjusted with 1 N. NaOH to approximately pH 6.2.

Cell suspensions from 48 hour cells grown on each of the above media were prepared and diluted with pH 6.0 buffer to 20 per cent transmission in the usual manner. A 5 ml. aliquot from each suspension was used to determine the dry weight. Aliquots of each suspension were run in the Warburg for 140 minutes. Because of the limitation in the number

of flasks which could be run on the Warburg at any one time, three different suspensions were required in order to run all of the amino acid solutions on cells from each medium. With each run on the Warburg two flasks were run to determine the endogenous respiration. To as many other duplicate flasks as were available (7 sets) 0.5 ml. of different amino acid solutions were added as substrate. This volume of amino acid solution gave a concentration of 0.25 mg. of amino acid per Warburg flask except in the case of tyrosine. The respiration in each case was calculated as microliters of oxygen per milligram of dried suspension. The results obtained are given in Tables 18, 19 and 20. Using the average substrate minus endogenous values from these tables the respiration was calculated to a basis of microliters of oxygen per milligram of nitrogen added as substrate per milligram of dried suspension. This was done in order to give a basis of comparison among the three media. These results are shown in Table 21.

In discussing these results it should be kept in mind that there are possibly three kinds of cells used in this experiment: cells after rapid growth and producing riboflavin (cells from the peptone medium); cells after moderate growth and not producing riboflavin (cells from the glutamic acid-arginine medium); and cells after moderate growth and producing riboflavin (cells from the glutamic acid-arginine-glycine medium).

A comparison of the substrate minus endogenous values for glycine, alpha-alanine and proline in Tables 18, 19 and 20 shows that the values decrease with decreased growth. The glycine data might be explained as follows: although glycine is present in the peptone medium, because of the rapid growth of the organisms with possible depletion of this amino

TABLE 18

RESPIRATION MEASUREMENTS ON SUSPENSIONS* OF CELLS
GROWN ON PEPTONE MEDIUM USING VARIOUS
AMINO ACIDS AS SUBSTRATE

Dry Weight	Oxygen absorbed/ mg. of cells/ 1h0 min.				
	Endogenous		Substrate		Substrate minus Endogenous Ave.
mg/ml	uL			uL	uL
2.20	49.4	Arginine		54.1	
	40.6			54.7	
Ave.	44.8		Ave.	54.4	9.6
1.87	43.2	Glycine		50.3	
	44.2			54.4	
Ave.	43.7		Ave.	52.4	8.7
		Cystine		53.2	
				49.5	
			Ave.	51.4	7.7
		Leucine		43.0	
				39.7	
			Ave.	41.4	0.0
		Threonine		46.0	
				44.4	
			Ave.	45.2	1.5
		alpha-Alanine		61.9	
				45.7	
			Ave.	53.8	10.1
		Lysine		50.8	
				49.4	
			Ave.	50.1	6.4
		Proline		48.2	
				54.0	
			Ave.	51.1	7.4
2.32	51.5	Tyrosine		51.9	
	38.0			53.2	
Ave.	44.8		Ave.	52.6	7.8

(Table continued on following page)

TABLE 18 (continued)

Dry Weight	Oxygen absorbed/ mg. of cells/ 140 min.			
	Endogenous	Substrate		Substrate minus Endogenous Ave.
mg/ml	uL	uL	uL	uL
		Tryptophan	43.8	
			42.6	
		Ave.	43.2	1.6
		Phenylalanine	43.8	
			48.3	
		Ave.	46.1	1.3
		Isoleucine	46.9	
			46.1	
		Ave.	46.5	1.7
		Serine	64.4	
			57.8	
		Ave.	61.1	16.3
		Glutamic Acid	56.8	
			48.8	
		Ave.	52.8	8.0
		Asparagine**	48.6	
			50.5	
		Ave.	49.6	4.8

*Suspensions were prepared from 48 hour cells and were diluted to 20 per cent transmission.
**Asparagine is a derivative of aspartic acid.

acid in the medium, glycine causes an increase in respiration when used as a substrate. There is a lesser increase in respiration from glycine for the slower growing glutamic acid-arginine cells because other amino acids have to be synthesized by the organism for glycine to be incorporated into proteins. If it is assumed that the cells from the glutamic acid-arginine-glycine medium are "saturated" with glycine, the lack of

TABLE 19

RESPIRATION MEASUREMENTS ON SUSPENSIONS* OF CELLS
GROWN ON GLUTAMIC ACID-ARGININE MEDIUM
USING VARIOUS AMINO ACIDS AS SUBSTRATE

Dry Weight	Oxygen absorbed/ mg. of cells/ 1h0 min. Endogenous	Substrate		Substrate minus Endogenous Ave.
mg/ml	uL		uL	uL
2.62	29.3	Arginine	31.0	
	24.1		29.0	
Ave.	26.7	Ave.	30.0	3.3
2.55	34.6	Glycine	43.1	
	34.5		38.0	
Ave.	34.6	Ave.	40.6	6.0
		Cystine	35.1	
			36.3	
		Ave.	35.7	1.1
		Leucine	37.3	
			32.9	
		Ave.	35.1	0.5
		Threonine	39.0	
			41.1	
		Ave.	40.1	5.5
		alpha-Alanine	41.3	
			----**	
		Ave.	41.3	6.7
		Lysine	43.5	
			44.7	
		Ave.	44.1	9.5
		Proline	41.7	
			38.0	
		Ave.	37.9	5.3
2.25	39.9	Tyrosine	45.5	
	37.9		42.9	
Ave.	38.9	Ave.	44.2	5.3

(Table continued on following page)

TABLE 19 (continued)

Dry Weight	Oxygen absorbed/ mg. of cells/ 1½0 min.			
	Endogenous	Substrate		Substrate minus Endogenous Ave.
mg/ml	uL		uL	uL
		Tryptophan	40.4	
			38.0	
		Ave.	39.2	0.3
		Phenylalanine	40.0	
			40.5	
		Ave.	40.3	1.4
		Isoleucine	35.6	
			36.8	
		Ave.	36.2	7.3
		Serine	45.3	
			46.5	
		Ave.	45.9	7.0
		Glutamic Acid	38.5	
			36.6	
		Ave.	37.6	0.0
		Asparagine***	49.2	
			44.4	
		Ave.	46.8	7.9

*Suspensions were prepared from 48 hour cells and were diluted to 20 per cent transmission.

**Sample was discarded because of a leak in the connections.

***Asparagine is a derivative of aspartic acid.

effect of glycine as substrate can be explained. Alpha-alanine and proline effects are also in proportion to the amount of growth, the largest effect on respiration being with the peptone cells and the least effect, with the glutamic acid-arginine-glycine cells. The respiration values for these three amino acids in Table 21 where values are calculated per milligram of nitrogen emphasizes this possible relationship between

TABLE 20

RESPIRATION MEASUREMENTS ON SUSPENSIONS* OF CELLS GROWN ON GLUTAMIC ACID-ARGININE-GLYCINE MEDIUM USING VARIOUS AMINO ACIDS AS SUBSTRATE

Dry Weight	Oxygen absorbed/ mg. of cells/ 1½ min. Endogenous	Substrate		Substrate minus Endogenous Ave.
mg/ml	uL		uL	uL
2.26	25.3	Arginine	33.3	
	20.7		33.7	
Ave.	23.0	Ave.	33.5	10.5
2.53	30.9	Glycine	26.3	
	28.0		---**	
Ave.	29.5	Ave.	26.3	0.0
		Cystine	30.0	
			34.2	
		Ave.	32.1	2.6
		Leucine	30.0	
			29.2	
		Ave.	29.6	0.1
		Threonine	29.7	
			29.3	
		Ave.	29.5	0.0
		alpha-Alanine	31.1	
			33.7	
		Ave.	32.4	2.9
		Lysine	32.1	
			35.3	
		Ave.	33.7	4.2
		Proline	33.6	
			33.2	
		Ave.	33.4	3.9
2.24	35.5	Tyrosine	44.3	
	35.0		40.3	
Ave.	35.3	Ave.	42.3	7.1

(Table continued on following page)

TABLE 20 (continued)

Dry Weight	Oxygen absorbed/ mg. of cells/ 140 min.			
	Endogenous	Substrate		Substrate minus Endogenous Ave.
mg/ml	uL		uL	uL
		Tryptophan	39.3	
			48.8	
		Ave.	44.1	8.9
		Phenylalanine	35.5	
			37.3	
		Ave.	36.4	1.2
		Isoleucine	35.8	
			36.7	
		Ave.	36.3	1.1
		Serine	51.5	
			48.8	
		Ave.	50.2	15.0
		Glutamic Acid	41.1	
			43.1	
		Ave.	42.1	6.9
		Asparagine***	48.9	
			52.5	
		Ave.	50.9	15.7

*Suspensions were prepared from 48 hour cells and diluted to 20 per cent transmission.
**Sample was discarded because of a leak in the connections.
***Asparagine is a derivative of aspartic acid.

respiration values and the type of cells as to growth. Stephens showed that riboflavin is formed when either proline or alpha-alanine was included in the glutamic acid-arginine medium. If riboflavin is formed in the Warburg with cells from this medium, it might also be a factor affecting respiration values.

In Table 21 serine appears to show very high respiration values.

TABLE 21

SUBSTRATE MINUS ENDOGENOUS RESPIRATION VALUES ON SUSPENSIONS* OF CELLS GROWN ON MEDIA WITH DIFFERENT NITROGEN SOURCES USING VARIOUS AMINO ACIDS AS SUBSTRATES

Substrate	Average substrate minus endogenous oxygen absorbed per mg. of cells per mg. of nitrogen per 140 min.		
	Peptone Medium	Glutamic acid-arginine Medium	Glutamic acid-arginine glycine Medium
	uL	uL	uL
Arginine	14.2	5.0	15.7
Glycine	18.6	13.0	0.0
Cystine	26.3	3.8	8.9
Leucine	0.0	1.7	0.4
Threonine	5.1	18.6	0.0
alpha-Alanine	25.7	17.0	7.4
Lysine	13.3	20.0	8.8
Proline	24.3	14.1	12.8
Tryptophan	4.7	0.9	26.0
Phenylalanine	6.1	6.6	5.6
Isoleucine	6.3	27.2	4.1
Serine	49.0	21.0	45.0
Glutamic Acid	33.6	0.0	29.0
Asparagine**	9.1	15.0	29.6

*Suspensions were prepared using 48 hour cells and were diluted to 20 per cent transmission.

**Asparagine is a derivative of aspartic acid.

Since values obtained with this amino acid with cells forming riboflavin are twice the value for cells not forming riboflavin (cells from the glutamic acid-arginine medium), it might be concluded that serine may be related directly or indirectly to riboflavin formation, although data obtained by Stephens, who added serine to this medium, showed that only small amounts of riboflavin were produced under the conditions he used.

Arginine also shows larger effects on respiration values in the media producing riboflavin than on the glutamic acid-arginine medium and might possibly be related in some way to riboflavin formation.

Frequently in physiological systems D forms of amino acids are inhibitory. Of the DL amino acids used, the relatively lower respiration values for threonine, tryptophan, phenylalanine and isoleucine might result from such inhibition. Apparently the D forms of serine and alpha-alanine are not inhibitory since these two amino acids showed among the largest increases in endogenous respiration observed in this experiment. It should be stated that the values of the DL amino acids calculated on a milligram of nitrogen basis, in Table 21, would be doubled if only the L form could be metabolized and if calculations were made on a milligram of metabolizable nitrogen basis.

If high respiration values on cells from the glutamic acid-arginine-glycine medium for the substances tested indicate a relationship to riboflavin formation, further studies on serine, glutamic acid, asparagine and tryptophan, which have high values as shown in Table 21, would be indicated.

13. Respiration studies on cells grown on different media with non-amino acid nitrogen sources as substrates. It has been reported by MacLaren that certain purines stimulate riboflavin production whereas certain pyrimidines inhibit it. It was thought that it might be of interest to study the effects of some of these compounds, added to the Warburg flask, on the respiration measurements of cell suspensions of A. gossypii grown on the basal medium with different sources of nitrogen added.

Accordingly, three different media were prepared exactly as in Experiment 12. Suspensions were prepared from 48 hour cells grown on each of these media. These suspensions were diluted with pH 6.0 buffer to 20 per cent transmission. A 10 ml. aliquot of each suspension was used for the determination of dry weight.

Solutions of the following nitrogen compounds were prepared by dissolving 50 mg. of each in sufficient pH 6.0 buffer and water to make 100 ml.: uracil, guanine hydrochloride, adenine sulfate, xanthine, diammonium phosphate and urea. Since all of the xanthine did not dissolve it was used as a saturated solution.

Duplicate aliquots from each of the above suspensions were run in the Warburg for 140 minutes without substrate to determine the endogenous respiration. To other duplicate samples, run in the same way, were added 0.5 ml.of each of the prepared solutions of nitrogen sources as substrate. Respiration measurements were calculated as microliters of oxygen per milligram of dried suspension. The results obtained are given in Table 22.

TABLE 22

RESPIRATION MEASUREMENTS ON SUSPENSIONS* OF 48 HOUR CELLS GROWN ON MEDIA WITH DIFFERENT NITROGEN SOURCES USING VARIOUS NON-AMINO ACID NITROGEN SOURCES AS SUBSTRATE

Substrate	Oxygen absorbed/ mg. of cells/ 140 min.		
	Peptone Medium	Glutamic acid-arginine Medium	Glutamic acid-arginine-glycine Medium
	uL	uL	uL
None (endogenous)	49.4	29.3	25.3
	40.6	24.1	20.7
Ave.	47.5	26.7	23.0
Uracil	50.3	33.0	22.8
	44.6	30.0	25.4
Ave.	47.5	31.5	24.1
Guanine.HCl	51.0	34.8	28.3
	44.1	33.7	30.7
Ave.	47.5	34.3	29.5
Adenine.SO_4	45.1	31.1	27.2
	41.7	29.0	29.2
Ave.	43.4	30.1	28.2
Xanthine	50.0	33.4	26.9
	54.5	33.6	30.0
Ave.	52.3	33.5	28.2
$(NH_4)_2HPO_4$	42.3	20.3	17.0
	43.4	22.3	22.5
Ave.	42.8	21.3	19.8
Urea	42.8	27.6	25.0
	41.1	27.2	22.6
Ave.	41.9	27.4	23.8

* Suspensions were diluted to 20 per cent transmission. Dry weight of peptone suspension was 2.20 mg/ml; dry weight of glutamic acid-arginine suspension was 2.62 mg/ml; and dry weight of glutamic acid-arginine-glycine suspension was 2.26 mg/ml.

Since only small or no effects on respiration occurred when the purines or the pyrimidine, uracil, were used as substrates, and since

little is known about the metabolism of these compounds in this organism, only speculation on the results can be made.

For cells grown on peptone medium only xanthine appears to increase respiration. It might be expected that cells grown on a relatively complete medium might show little effect of the addition of purines in small amounts, for if they are formed in the cells in normal metabolism on peptone, they may already be present in a concentration for maximal rate of oxidation. The increase in respiration with xanthine as substrate might result from an oxidative pathway that rapidly removes xanthine. A mechanism of this type may be necessary to avoid the accumulation of xanthine in normal metabolism for it is an intermediate in purine catabolism, that is, adenine is hydrolyzed, then oxidized to xanthine, and guanine is hydrolyzed to xanthine, when the appropriate enzymes are available.

By the same reasoning as above, it might be assumed that purines as substrates would have the effect of increasing the respiration of cells grown on the less complete media, glutamic acid-arginine and glutamic acid-arginine-glycine, in which it might be expected that smaller amounts of these purines would be synthesized.

With the cells from the two media inhibiting or delaying growth, guanine and xanthine cause greater increases in respiration than the other compounds tested. As mentioned above, the catabolism of guanine involves its conversion to xanthine. Thus it might be expected that guanine would cause effects similar to xanthine.

With regard to the pyrimidine uracil, it caused an appreciable increase in respiration only with the cells from the glutamic acid-arginine

medium, the medium which apparently does not provide suitable conditions for the formation of riboflavin. An investigation of uracil as an intermediate in riboflavin formation might yield interesting results.

The other two compounds, diammonium phosphate and urea, appear to inhibit endogenous respiration in all three types of cells used.

14. Effect of cold homogenization on respiration measurements of cells. It was noticed that during a long period of use the Waring blendor becomes quite warm. The decrease in respiration previously obtained with homogenization may have been due to the heat rather than to mechanical destruction of the cells by the blendor action. In an effort to determine whether different results would be obtained if the blendor and cells were kept cold during the period of homogenization, a suspension of 48 hour cells, grown on the basal medium with 0.25 per cent added peptone, was prepared. The Waring blendor was cooled before use by placing it in a bucket of crushed ice and water. The suspension was then placed in the blendor and homogenized, aliquots being removed after 5, 10, 20 and 60 seconds of homogenization. The Waring blendor was kept cold throughout the homogenization period by placing it in the bucket of crushed ice and water for short periods while each aliquot was being removed. Each aliquot was diluted with pH 6.0 buffer to a turbidity corresponding to 20 per cent transmission and placed in a 125 ml. Erlenmeyer flask which contained enough glass beads to completely cover the bottom of the flask. A 6 ml. aliquot of each suspension was used for the determination of dry weight. Duplicate samples from each suspension were run with 0.5 ml. of 0.1 M. glucose as substrate and without substrate to determine the endogenous respiration. All were run for 140 minutes in the Warburg. The respiration

was calculated as microliters of oxygen per milligram of dried cell suspension. The results are given in Table 23.

TABLE 23

RESPIRATION MEASUREMENTS ON A SUSPENSION* AFTER DIFFERENT PERIODS OF COLD HOMOGENIZATION

Time of homogenization	Dry weight	Oxygen absorbed/ mg. of cells/ 1h0 min. Endogenous	Substrate**	Substrate minus Endogenous Ave.
sec.	mg/ml	uL	uL	uL
5	2.h3	28.0	36.6	
		26.1	35.2	
	Ave.	27.1	35.9	8.8
10	2.87	18.0	20.5	
		17.6	20.7	
	Ave.	17.8	20.6	2.8
20	1.73	22.1	2h.8	
		21.h	25.9	
	Ave.	21.8	25.h	3.6
60	1.70	21.8	2h.0	
		19.0	----***	
	Ave.	20.h	2h.0	3.6

*Suspensions were prepared from h8 hour cells and diluted to 20 per cent transmission.
**Substrate consisted of 0.5 ml. of 0.1 M. glucose per Warburg flask.
***Sample discarded because of a leak in the connections.

A comparison of these results with those in Table 1h shows that the same sort of decrease in both endogenous respiration and respiration in the presence of glucose is obtained over the 10 to 60 second homogenization period in both cases. The major difference between the two sets of data is the value obtained for the substrate minus endogenous values.

These values from the homogenization where the blendor was kept warm show a decrease over the 10 to 60 second range of homogenization whereas the values from the cold homogenization remain approximately constant over this same range. Apparently the decrease in respiration obtained with the previous 60 second homogenization when the blendor was not kept cold, was due to temperature effects on the cells rather than to cell disintegration due to mechanical effects of homogenization. The fact that the readings from 10 to 60 seconds homogenization are relatively constant may indicate that the higher reading obtained with the 5 second homogenization may be due to incomplete disruption of the aggregates of cells with occluded medium which could affect the values obtained. The data indicate that any time of homogenization between 10 and 60 seconds would be satisfactory if the blendor is cooled. Thus all subsequent suspensions were homogenized for 10 seconds in the above manner and glass beads were added to the suspension to aid in obtaining more uniform aliquots.

15. Effect of riboflavin on respiration measurements on a suspension of cells grown on peptone medium. To determine what effect riboflavin itself had on respiration values aliquots of the same washed suspension of 48 hour cells adjusted to 20 per cent transmission used in Experiment 5 were run for 140 minutes in the Warburg both in the presence and absence of glucose and in the presence of riboflavin alone and riboflavin with glucose. Warburg flasks were prepared in the usual manner in duplicate for both the endogenous and glucose respiration measurements. In the same way flasks were set up like those for the endogenous respiration except that 0.5 ml. of a solution containing 25 ug. of riboflavin

per ml. was added to each flask. Similarly, two flasks were prepared like those for the glucose respiration except that each flask contained the same added amount of riboflavin as above. The results obtained are given in Table 24.

TABLE 24

EFFECT OF ADDED RIBOFLAVIN ON RESPIRATION MEASUREMENTS ON A SUSPENSION* OF 48 HOUR CELLS GROWN ON PEPTONE MEDIUM

Substrate		Oxygen absorbed/ mg. of cells/ 140 min.	Substrate minus endogenous Ave.
		uL	uL
None (endogenous)		11.9	
		13.1	
	Ave.	12.5	
0.5 ml. of 0.1 M. glucose		21.0	
		20.2	
	Ave.	20.6	8.1
0.5 ml. of riboflavin solution**		24.9	
		24.7	
	Ave.	24.8	12.3
0.5 ml. of 0.1 M. glucose and 0.5 ml. of riboflavin solution**		32.5	
		30.6	
	Ave.	31.6	19.1

*Suspension was diluted to 20 per cent transmission. Dry weight was 2.79 mg/ml of suspension.
**Riboflavin solution contained 25 ug. riboflavin per ml.

The results indicate that riboflavin causes a higher oxygen uptake than glucose in the concentrations used. The value for the oxygen absorbed in the glucose-riboflavin flasks is about the same as the sum of the two separate values. This might lead to the conclusion that

glucose and riboflavin metabolism are not interrelated, at least under the conditions studied. It would appear that riboflavin in excess of that already present in enzyme systems in the cells is oxidized by 48 hour peptone cells. If such is the case it might account for why riboflavin does not accumulate much until after the rapid growth phase. However, the results might just as well be interpreted as showing a stimulation of endogenous respiration. In this interpretation it would be assumed that riboflavin present in the cells is the factor limiting the rate of endogenous respiration.

16. Respiration measurements at pH 8.0 on suspensions of cells grown on different media using various amino acids as substrate. In the study of amino acids in Experiment 12, a pH 6.0 buffer was used in the determination of the respiration values since this pH is optimal for the growth of the organism. It was felt that if amino acids played any large part in the growth or production of riboflavin by A. gossypii the effects would take place at the pH of optimal growth. Since amino acid oxidases are more active at pH 8.0, a second set of media was prepared in the same manner as in the above experiment. It was also felt that a better idea of the amino acid oxidative activity of the cells could be obtained if the cells were studied after 48 hours incubation, when there should have been large quantities of the supplied nutrients remaining, and after 96 hours when the medium should have been somewhat depleted of nutrients. The cell suspensions were prepared in the usual manner except that they were diluted with pH 8.0 buffer to a transmission between 10 and 20 per cent, depending on the number of cells available. a 5 ml. aliquot of each suspension was used for the determination of dry

weights.

The following amino acids were prepared in a concentration of 0.01 M. by dissolving them in pH 8.0 buffer; glycine, DL-alpha-alanine, L-lysine, L-glutamic acid, L-arginine monohydrochloride, DL-threonine, DL-isoleucine and L-asparagine (a derivative of aspartic acid). Duplicate aliquots of each suspension were run without substrate to determine the endogenous respiration and with 0.5 ml. of 0.1 M. glucose as substrate. Quadruplicate aliquots of the suspensions were run with 0.5 ml. of 0.01 M. amino acid solution as substrate, half of these aliquots also containing 0.5 ml. of 0.1 M. glucose as additional substrate. Due to limitations of space on the Warburg apparatus, it was necessary to run four cell suspensions with each age of cells for each medium. All samples were run in the Warburg for 140 minutes and the respiration was calculated as microliters of oxygen absorbed per milligram of dried suspension. The results are given in Tables 25 through 30.

The results given in Tables 25 through 30 are unfortunately complicated by being run at an unfavorable pH for overall metabolism. This is shown by the unusually low endogenous respiration values for both the 48 hour and 96 hour cells from all of the media. The possible advantage gained by making measurements at a pH favorable for amino acid oxidases, as mentioned earlier, was more than offset by the apparent effect on overall metabolism.

Although the effects of the various substrates tried are small, the values for glycine, alpha-alanine and proline on the 48 hour cells appear to show the same relative effects as in Experiment 12. Each of these amino acids has its greatest effect on the peptone cells and its

TABLE 25

RESPIRATION MEASUREMENTS AT pH 8.0 ON 48 HOUR PEPTONE CELL SUSPENSIONS USING GLUCOSE AND VARIOUS AMINO ACIDS AS SUBSTRATES

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 140 min.					
		Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
2.42	19	14.6	31.2	Glycine	27.8	34.5	
		18.5	33.7		24.1	34.0	
	Ave.	16.6	32.5		26.0	34.3	9.4
				alpha-Alanine	25.7	29.2	
					24.9	28.7	
				Ave.	25.3	29.0	8.7
				Lysine	21.6	28.2	
					23.3	29.8	
				Ave.	22.5	29.0	5.9
2.66	20	19.7	27.7	Proline	24.0	33.3	
		17.7	29.5		20.2	29.2	
	Ave.	18.7	28.6		22.1	30.1	3.4
				Tyrosine	21.1	27.0	
					20.6	27.2	
				Ave.	20.9	27.1	2.2

(Table continued on following page)

TABLE 25 (continued)

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 1h0 min. Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
				Serine	15.0	28.0	
					21.8	25.5	
				Ave.	19.9	26.8	0.0
2.13	20	11.3	18.7	Glutamic acid	17.6	20.6	
		13.1	21.0		16.1	23.6	
	Ave.	12.2	19.9	Ave.	16.9	22.1	4.7
				Arginine	15.7	11.0	
					15.1	18.3	
				Ave.	15.4	14.7	3.2
				Threonine	11.4	16.0	
					12.1	17.6	
				Ave.	11.8	16.8	0.0
1.90	20	13.3	22.1	Asparagine*	15.3	22.5	
		16.7	21.7		13.7	24.6	
	Ave.	15.0	21.7	Ave.	14.5	23.6	0.0
				Isoleucine	16.8	19.9	
					16.0	24.4	
				Ave.	16.4	22.2	1.4

*Asparagine is a derivative of aspartic acid.

TABLE 26

RESPIRATION MEASUREMENTS AT pH 8.0 ON 96 HOUR PEPTONE CELL SUSPENSIONS USING GLUCOSE AND VARIOUS AMINO ACIDS AS SUBSTRATES

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 1½0 min.					
		Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
4.02	12	10.3	12.6	Glycine	10.8	14.2	
		7.9	14.2		10.9	14.2	
	Ave.	9.1	13.4		10.9	14.2	1.8
				alpha-Alanine	10.2	10.0	
					10.9	12.7	
				Ave.	10.6	11.4	1.5
				Lysine	10.5	13.3	
					10.2	13.2	
				Ave.	10.4	13.3	1.3
4.96	10	10.0	14.0	Proline	7.8	14.6	
		9.0	14.3		11.1	15.0	
	Ave.	9.5	14.2		9.5	14.8	0.0
				Tyrosine	10.7	11.5	
					5.6	13.5	
				Ave.	8.2	12.5	0.0

(Table continued on following page)

TABLE 26 (continued)

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 1h0 min.					
		Endogenous	Glucose (I) substrate	Amino acid (II) substrates		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
				Serine	11.3	12.8	
					9.7	12.3	
				Ave.	10.5	12.5	1.0
5.15	10	8.8	1h.6	Glutamic acid	13.8	16.2	
		--- *	13.1		12.8	17.0	
		Ave.	13.9		13.3	16.6	h.5
				Arginine	11.8	13.7	
					12.0	13.6	
				Ave.	11.9	13.7	3.1
				Threonine	11.h	15.6	
					12.5	12.1	
				Ave.	12.0	13.9	3.2
h.21	11	1h.1	2h.5	Asparagine**	19.3	25.h	
		13.7	23.3		18.8	2h.8	
		Ave. 13.9	23.9		19.1	25.1	5.2
				Isoleucine	15.5	21.9	
					13.7	22.0	
				Ave.	1h.6	22.0	0.7

*Sample was discarded because of a leak in the connections.

**Asparagine is a derivative of aspartic acid.

TABLE 27

RESPIRATION MEASUREMENTS AT pH 8.0 ON 48 HOUR GLUTAMIC ACID-ARGININE CELL SUSPENSIONS USING GLUCOSE AND VARIOUS AMINO ACIDS AS SUBSTRATES

Dry weight	Transmission		Oxygen absorbed/ mg. of cells/ 140 min.					
		Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
6.00	11	11.1	14.3	Glycine	10.2	15.7	
		11.2	12.6		10.8	17.0	
	Ave.	11.2	13.5		10.6	16.4	0.0
				alpha-Alanine	11.2	13.5	
					10.5	14.6	
				Ave.	10.9	14.1	0.0
				Lysine	11.5	14.1	
					11.3	13.5	
				Ave.	11.4	13.8	0.2
2.49	20	4.5	7.4	Proline	8.7	7.9	
		5.1	---*		6.6	9.5	
	Ave.	4.8			7.7	8.7	2.9
				Tyrosine	4.3	6.8	
					3.4	8.1	
				Ave.	3.8	7.5	0.0

(Table continued on following page)

TABLE 27 (continued)

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 1½0 min.					
		Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
				Serine	6.1	8.4	
					5.8	7.0	
				Ave.	6.0	7.7	1.2
2.00	20	10.6	19.2	Glutamic acid	16.7	20.6	
		7.0	18.5		17.9	23.4	
	Ave.	8.8	18.9		17.3	22.0	8.5
				Arginine	15.2	15.0	
					16.8	8.7	
				Ave.	16.0	11.9	7.2
				Threonine	12.8	17.1	
					12.9	16.6	
				Ave.	12.9	16.9	4.1
2.03	20	13.1	20.0	Asparagine**	15.7	19.3	
		10.8	19.9		14.9	20.3	
	Ave.	12.0	20.0		15.3	19.8	3.3
				Isoleucine	14.2	15.4	
					9.0	20.0	
				Ave.	11.6	17.7	0.0

*Sample was discarded because of a leak in the connections.

**Asparagine is a derivative of aspartic acid.

TABLE 28

RESPIRATION MEASUREMENTS AT pH 8.0 ON 96 HOUR GLUTAMIC ACID-ARGININE CELL SUSPENSIONS USING GLUCOSE AND VARIOUS AMINO ACIDS AS SUBSTRATES

Dry weight	Transmission		Oxygen absorbed/ mg. of cells/ 1½0 min. Endogenous	Glucose (I) substrate	Amino Acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent		uL	uL		uL	uL	uL
3.07	15		6.6	7.7	Glycine	6.6	7.4	
			5.0	7.5		5.4	7.2	
		Ave.	5.8	7.6		6.0	7.3	0.2
					alpha-Alanine	5.5	5.1	
						5.4	6.1	
					Ave.	5.5	5.6	0.0
					Lysine	5.8	7.2	
						5.8	7.1	
					Ave.	5.8	7.2	0.0
4.62	12		7.6	9.7	Proline	9.6	9.7	
			7.2	11.0		7.4	10.6	
		Ave.	7.4	10.4		8.5	10.2	1.1
					Tyrosine	7.9	9.2	
						6.8	9.9	
					Ave.	7.4	9.6	0.0

(Table continued on following page)

TABLE 28 (continued)

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 1½0 min. Endogenous	Glucose (I) substrate	Amino acid (II) substrate	I and II Substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
				Serine	5.8	8.4	
					6.3	---*	
				Ave.	6.1		0.0
3.00	15	8.5	13.4	Glutamic Acid	11.6	13.6	
		9.3	14.9		11.0	13.2	
	Ave.	8.9	14.2		11.3	13.4	2.4
				Arginine	8.6	10.4	
					7.9	9.2	
				Ave.	8.3	9.8	0.0
				Threonine	9.3	11.9	
					11.3	11.6	
				Ave.	10.3	11.8	1.4
6.84	13	2.0	4.5	Asparagine**	4.4	5.3	
		2.7	5.4		4.4	5.8	
	Ave.	2.4	5.0		4.4	5.6	2.0
				Isoleucine	5.8	4.8	
					4.5	4.2	
				Ave.	5.2	4.5	2.8

*Samples discarded because of a leak in the connections.

**Asparagine is a derivative of aspartic acid.

TABLE 29

RESPIRATION MEASUREMENTS AT pH 8.0 ON 48 HOUR GLUTAMIC ACID-ARGININE-GLYCINE CELL SUSPENSIONS USING GLUCOSE AND VARIOUS AMINO ACIDS AS SUBSTRATES

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 140 min.					
		Endogenous	Glucose (I) substrate	Amino Acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
3.27	17	10.8	14.9	Glycine	10.6	10.6	
		10.1	14.5		10.5	13.5	
	Ave.	10.5	14.7	Ave.	10.6	12.1	0.1
				alpha-Alanine	7.7	9.6	
					8.5	12.7	
				Ave.	8.1	11.2	0.0
				Lysine	8.5	13.9	
					11.4	14.1	
				Ave.	10.5	14.0	0.0
2.18	20	10.4	11.5	Proline	8.6	13.8	
		7.6	12.7		5.7	13.1	
	Ave.	9.0	12.1		7.2	13.5	0.0
				Tyrosine	5.6	7.8	
					8.9	13.3	
				Ave.	7.3	10.6	0.0

(Table continued on following page)

TABLE 29 (continued)

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 1½0 min. Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
				Serine	7.0	11.6	
					14.0	16.4	
				Ave.	10.5	14.0	1.5
2.63	20	12.9	20.2	Glutamic acid	30.8	29.2	
		12.1	15.2		25.1	28.9	
		Ave. 12.5	17.7		28.0	29.1	15.5
				Arginine	15.6	18.3	
					7.5	22.0	
				Ave.	12.1	20.2	0.0
				Threonine	17.9	19.7	
					----*	17.6	
				Ave.		18.7	5.4
2.21	20	14.4	19.0	Asparagine**	11.1	17.7	
		----*	19.7		11.8	18.6	
		Ave.	19.4		11.5	18.2	0.0
				Isoleucine	15.1	13.6	
					9.5	18.3	
				Ave.	12.3	16.0	0.0

*Sample was discarded because of a leak in the connections.

**Asparagine is a derivative of aspartic acid.

TABLE 30

RESPIRATION MEASUREMENTS AT pH 8.0 ON 96 HOUR GLUTAMIC ACID-ARGININE-GLYCINE CELL SUSPENSIONS USING GLUCOSE AND VARIOUS AMINO ACIDS AS SUBSTRATES

Dry weight	Transmission	Oxygen absorbed/ mg. of cells/ 1h0 min.					
		Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent	uL	uL		uL	uL	uL
3.01	16	11.3	14.3	Glycine	13.2	15.5	
		8.5	15.3		12.5	15.8	
	Ave.	9.9	14.8	Ave.	12.9	15.7	3.0
				alpha-Alanine	11.6	12.9	
					8.4	15.4	
				Ave.	10.0	14.2	0.1
				Lysine	9.7	13.8	
					10.5	12.0	
				Ave.	10.1	12.9	0.2
4.71	10	13.2	25.4	Proline	15.1	26.2	
		12.1	26.2		15.2	28.5	
	Ave.	12.7	25.8		15.2	27.4	2.5
				Tyrosine	14.8	28.8	
					15.5	27.9	
				Ave.	15.2	28.4	2.5

(Table continued on following page)

TABLE 30 (continued)

Dry weight	Transmission		Oxygen absorbed/ mg. of cells/ 1½0 min. Endogenous	Glucose (I) substrate	Amino acid (II) substrate		I and II substrates	II substrate minus endogenous Ave.
mg/ml	per cent		uL	uL		uL	uL	uL
					Serine	17.0	30.9	
						16.8	29.1	
					Ave.	16.9	30.0	4.2
3.56	13		13.9	19.9	Glutamic acid	21.1	23.4	
			13.9	19.9		21.6	28.5	
		Ave.	13.9	19.9		21.4	26.0	7.5
					Arginine	20.0	21.9	
						19.3	22.4	
					Ave.	19.7	22.2	5.8
					Threonine	19.7	21.7	
						25.8	22.3	
					Ave.	22.8	22.0	8.9
3.41	12		8.7	11.6	Asparagine**	8.5	11.9	
			----*	12.3		6.5	12.5	
		Ave.		12.0		7.5	12.2	0.0
					Isoleucine	9.8	11.1	
						9.4	11.5	
					Ave.	9.6	11.3	0.9

*Sample discarded because of a leak in the connections.

**Asparagine is a derivative of aspartic acid.

smallest effect on the glutamic acid-arginine-glycine cells. In addition, the amino acids lysine and tyrosine are similar, in their effects, to the above three amino acids.

These results indicate that both the 48 and 96 hour cells in each medium show a greater oxidative activity when the amino acid is the sole substrate. Several of the amino acids oxidize in the absence of glucose and are apparently not used when glucose is present. In a very few cases the oxidation of glucose is stimulated by the presence of an amino acid which has no effect on the endogenous respiration; for example, asparagine has a greater stimulative effect on glucose oxidation in the 48 hour peptone cells than it has on endogenous respiration, whereas in the 96 hour peptone cells it stimulates the endogenous respiration.

The largest single effect is obtained with glutamic acid on endogenous and endogenous plus glucose in the 48 hour glutamic acid-arginine-glycine cells. Glutamic acid has appeared to be directly related to growth and indirectly, to riboflavin formation in several of the experiments. In all of the media except those containing peptone, glutamic acid has been the main source of nitrogen.

17. Effect of cell extracts added to the Warburg flasks on respiration measurements on suspensions of cells grown on media with different sources of nitrogen. Stephens showed that when small amounts of water extracts of cells grown on peptone and yeast extract medium were added to a medium containing glutamic acid, arginine and glycine, a much greater production of riboflavin occurred earlier than without the addition of this cell extract. The effect of stimulation of riboflavin formation was found by him to be in the heavier material which did not

move on paper strips when the extracts were chromatographed. It was felt that studies of respiration measurements made by adding the whole cell extract, and chromatographically separated fractions of it, to cells grown on the basal medium with different sources of nitrogen, and on an enriched medium, might be of interest.

Washed suspensions of 24 and 48 hour cells, grown on a peptone-yeast extract medium containing 2.0 per cent glucose, 0.5 per cent yeast extract and 0.25 per cent peptone, were prepared and diluted with pH 6.0 buffer to 10 per cent transmission. A 5 ml. aliquot of each suspension was used for the determination of dry weight. Duplicate samples from each of these suspensions were run for 140 minutes in the Warburg to obtain the endogenous respiration. Duplicate samples from each suspension were also run with each of the following substrates: 0.5 ml. of 0.1 M. glucose, 0.1 ml. of acetone extract of cells*, 0.1 ml. of acetone extract of cells with 0.5 ml. of 0.1 M. glucose, 0.2 ml. of water extract of cells* and 0.2 ml. of water extract of cells with 0.5 ml. of 0.1 M. glucose. Single flasks were run for both the 24 and 48 hour cell suspensions using 0.05 ml. of acetone extract as substrate. All of the flasks with 24 hour cells were run at the same time in the Warburg and with them were run duplicate flasks containing 0.1 ml. of the acetone extract without cells to serve as controls. To serve as controls for the water extract, duplicate flasks containing 0.2 ml. of the water extract without cells were run at the same time as the 48 hour cells. All samples were run in the Warburg for 140 minutes. The respiration was calculated as microliters of oxygen absorbed per milligram of dried suspension. The results are given in Table 31.

*Acetone extracts and water extracts of cells were furnished by R. L. Stephens of the Chemistry Department at the University of Florida.

TABLE 31

RESPIRATION MEASUREMENTS ON PEPTONE-YEAST EXTRACT CELL SUSPENSIONS* WITH CELL EXTRACTS ADDED AS SUBSTRATE

Substrate	Oxygen absorbed/ mg. of cells/ 1½0 min. 24 hour cells	48 hour cells
	uL	uL
None (endogenous values)	54.0	27.7
	57.0	28.0
Ave.	55.5	27.9
0.5 ml. of 0.1 M. glucose	143.6	70.8
	151.6	78.4
Ave.	147.6	74.6
0.1 ml. of acetone extract	87.5	55.2
	84.7	53.3
Ave.	86.1	54.3
0.1 ml. of acetone extract	165.0	92.6
and 0.5 ml. of 0.1 M. glucose	180.1	101.1
Ave.	172.6	96.9
0.05 ml. of acetone extract	76.4	39.9
	---**	46.0
Ave.		43.0
0.2 ml. of water extract	166.1	94.6
	156.7	103.1
Ave.	161.4	98.9
0.2 ml. of water extract	239.2	117.2
and 0.5 ml. of 0.1 M. glucose	220.9	115.3
Ave.	230.1	116.3
0.1 ml. of acetone extract	15.6	
	15.3	
Ave.	15.5	
0.2 ml. of water extract		0.0
		0.0
	Ave.	0.0

*Suspensions were diluted to 10 per cent transmission. Dry weight of the 24 hour suspension was 4.83 mg./ml. Dry weight of the 48 hour suspension was 4.34 mg./ml.

**A duplicate sample was not run because of space limitations on the Warburg apparatus.

The data in Table 31 show that cells grown on peptone and yeast extract medium have a much higher respiration at 24 hours than they have at 48 hours, which is to be expected, because cells grown on an enriched medium would probably reach the end of the rapid growth phase earlier than those grown on the other media used. For both 24 and 48 hour cells the addition of 0.1 ml. of acetone extract of cells caused an increase in respiration in the absence of glucose. These respiration values are much higher than any of the values obtained on cells from different media both with or without substrates. This increase appears to be due to a stimulation of endogenous respiration. The probability of substances being oxidized in the extract accounting for the large increase in respiration is considered to be unlikely because only relatively small amounts of substances are present in the extract. The respiration obtained by adding both glucose and acetone extract is slightly less than the sum of the separate values. The same effects are seen in the results when the water extract is added.

The acetone extract in the absence of cells shows a slight respiration value which may perhaps be due to the presence of enzymes and/or riboflavin in the extract. The water extract, however, which also contains riboflavin, and may contain enzymes, does not show this effect.

The water extract added to the flasks has a greater effect than the acetone extract. The difference in results obtained with the two extracts may be due to differences in amounts of substances present. However, since both extracts exert this effect, whereas only the water extract stimulates the formation of riboflavin, according to Stephens, it may be that the larger increase in respiration obtained with the water

extract was related to the metabolic processes involved in the formation of riboflavin.

The following work deals with the effect of different portions of the chromatograms of the water cell extract on respiration measurements. Four paper chromatograms* of the water extract of cells, using butanol-acetic acid solvent, were air dried and cut into four portions designated as A, B, C and D. The A portion, which consisted of the bottom 1½ inch of the paper strips, included the non-moving residue of the extract, which Stephens had shown to stimulate the early formation of riboflavin in the glutamic acid-arginine-glycine medium. The B portion consisted of the next 1½ inch of the strips. The C portion, which consisted of the next 1½ inch of the strips contained the riboflavin. The D portion consisted of the rest of the strips, about 4 inches. Some filter paper strips treated only with butanol and acetic acid and dried were used as a control. The A, B, C and D portions of the strips (four of each portion were pooled) of the chromatograms and the control strips were homogenized separately in the Waring blendor for 1 minute with 25 ml. portions of pH 6.0 buffer to prepare extracts for testing.

Washed suspensions of 24 and 48 hour cells, grown both on the basal medium with 0.25 per cent added peptone and on the enriched peptone-yeast extract medium, prepared as above, were made to study the effects of these portions of the chromatograms on respiration measurements. These suspensions were homogenized for 10 seconds in the Waring blendor and were diluted to 10 per cent transmission with pH 6.0 buffer. A 5 ml.

*The paper chromatograms of the water cell extract were furnished by R. L. Stephens.

aliquot of each suspension was used to determine the dry weight. Because of limitations of Warburg space, it was necessary to use a different suspension of cells for each of the A, B, C and D portions of the chromatograms. Each Warburg run was set up using duplicate aliquots of the suspensions for endogenous respiration and for each of the following: 1.0 ml. of treated paper control extract; 0.5 ml. of 0.1 M. glucose; 0.5 ml. of 0.1 M. glucose with 1.0 ml. of treated paper control extract; 0.25 ml. of chromatogram extract; 0.25 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose; 1.0 ml. of chromatogram extract; and 1.0 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose. All samples were run in the Warburg for 140 minutes and the respiration measurements were calculated as microliters of oxygen absorbed per milligram of dried suspension. The results obtained are given in Tables 32 - 35.

The data in Tables 32 - 35 show that in most cases the endogenous respiration and the respiration in the presence of glucose are increased by the treated paper control extract, which may have contained some butanol-acetic acid solvent. However, effects from all the fraction extracts and the treated paper control extracts are considered to be too small to be of any significance. Along with the lack of appreciable effects are appreciable variations in results that may be due to different adsorption effects from the ground filter paper in different flasks.

Three possible explanations are offered for the difference between the large effects from entire extracts and the small effects from different chromatographic portions of the extract; the amount of active substances in the portions of the chromatograms were used in too low

TABLE 32

RESPIRATION MEASUREMENTS ON 24 HOUR PEPTONE CELL SUSPENSIONS*
WITH CHROMATOGRAPHED CELL EXTRACT ADDED AS SUBSTRATE

Substrate	Suspension:	Oxygen absorbed per mg. of cells per 140 minutes 1	2	3	4
		uL	uL	uL	uL
None (endogenous values)		40.1	36.7	26.5	23.1
		31.2	39.0	27.6	15.3
	Ave.	35.7	37.9	27.1	19.2
1.0 ml. of treated paper control extract		38.6	37.4	33.7	27.4
		39.9	36.5	31.8	21.4
	Ave.	39.3	32.0	32.8	24.4
0.5 ml. of 0.1 M. glucose		59.5	56.0	46.4	25.0
		59.7	58.0	47.3	26.4
	Ave.	59.6	57.0	46.9	25.7
0.5 ml. of 0.1 M. glucose with 1.0 ml. of treated paper control extract		61.6	55.0	42.3	23.5
		59.8	55.2	43.9	22.1
	Ave.	60.7	55.1	43.1	22.8
	With chromatogram extract:	A	B	C	D
0.25 ml. of chromatogram extract		37.0	32.8	30.8	29.0
		38.6	39.3	34.6	22.1
	Ave.	37.8	36.1	32.7	25.6

(Table continued on following page)

TABLE 32 (continued)

Substrate	With chromatogram extract:	Oxygen absorbed per mg. of cells per 1h0 minutes A	B	C	D
		uL	uL	uL	uL
0.25 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose		56.h	57.2	h6.0	26.8
		60.3	58.9	h0.0	27.1
	Ave.	58.h	58.1	h3.0	27.0
1.0 ml. of chromatogram extract		3h.8	h0.8	3h.h	27.7
		35.6	h2.5	31.2	27.8
	Ave.	35.2	h1.7	32.8	27.8
1.0 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose		55.6	51.5	hh.0	30.6
		52.1	5h.7	52.3	31.3
	Ave.	5h.h	53.1	h8.7	31.0

*Suspensions were diluted to 10 per cent transmission. Dry weights of suspensions 1, 2, 3 and h were h.56, 5.6h, h.30 and 5.08 mg. per ml., respectively.

TABLE 33

RESPIRATION MEASUREMENTS ON 48 HOUR PEPTONE CELL SUSPENSIONS*
WITH CHROMATOGRAPHED CELL EXTRACT ADDED AS SUBSTRATE

Substrate	Suspension:	Oxygen absorbed per mg. of cells per 140 minutes 1	2	3	4
		uL	uL	uL	uL
None (endogenous values)		34.8	64.2	41.5	21.5
		35.0	64.2	39.2	19.3
	Ave.	34.9	64.2	40.4	20.4
1.0 ml. of treated paper control extract		37.7	71.3	41.9	21.1
		40.3	73.0	42.9	23.2
	Ave.	39.0	72.2	42.4	22.2
0.5 ml. of 0.1 M. glucose		50.2	76.3	56.0	29.8
		48.5	70.5	48.3	31.0
	Ave.	49.4	73.4	52.2	30.1
0.5 ml. of 0.1 M. glucose with 1.0 ml of treated paper control extract		49.5	77.3	49.0	28.8
		51.0	71.1	56.0	24.7
	Ave.	50.3	74.2	52.5	26.8
	With chromatogram extract:	A	B	C	D
0.25 ml. of chromatogram extract		35.5	57.2	31.6	17.0
		35.6	60.4	32.3	22.7
	Ave.	35.6	58.8	32.0	19.9

(Table continued on following page)

TABLE 33 (continued)

Substrate	With chromatogram extract:	Oxygen absorbed per mg. of cells per 1h0 minutes A	B	C	D
		uL	uL	uL	uL
0.25 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose		51.3	80.0	h3.5	27.5
		h9.2	81.3	36.9	3h.8
	Ave.	50.3	80.7	h0.2	31.2
1.0 ml of chromatogram extract		38.2	71.7	35.6	2h.1
		36.3	71.0	33.7	23.7
	Ave.	37.3	71.h	3h.7	23.9
1.0 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose		51.1	85.0	h8.0	32.h
		h2.3	87.0	h8.8	36.h
	Ave.	h6.7	86.0	h8.h	3h.h

*Suspensions were diluted to 10 per cent transmission. Dry weights of suspensions 1, 2, 3 and h were h.97, 3.99, h.56 and 5.06 mg. per ml., respectively.

TABLE 34

RESPIRATION MEASUREMENTS ON 24 HOUR PEPTONE-YEAST EXTRACT CELL SUSPENSIONS*
WITH CHROMATOGRAPHED CELL EXTRACT ADDED AS SUBSTRATE

Substrate	Suspension:	Oxygen absorbed per mg. of cells per 140 minutes			
		1	2	3	4
		uL	uL	uL	uL
None (endogenous values)		44.7	97.8	54.9	57.0
		36.6	85.4	49.2	54.0
	Ave.	40.7	91.6	52.1	55.5
1.0 ml. of treated paper control extract		57.2	197.8**	64.5	63.1
		57.8	176.6	64.4	62.3
	Ave.	57.5	187.2	64.5	62.7
0.5 ml. of 0.1 M. glucose		91.0	100.9**	95.4	83.5
		90.0	94.8	88.2	72.2
	Ave.	90.5	97.9	91.8	77.9
0.5 ml. of 0.1 M. glucose with 1.0 ml. of treated paper control extract		94.7	202.3	104.3	84.9
		76.9	153.3	105.1	81.2
	Ave.	85.8	177.8	104.7	83.1
	With chromatogram extract:	A	B	C	D
0.25 ml. of chromatogram extract		53.9	82.6	54.9	54.6
		58.0	87.9	56.7	54.1
	Ave.	56.0	85.3	55.8	54.4

(Table continued on following page)

TABLE 34 (continued)

Substrate With chromatogram extract:		Oxygen absorbed per mg. of cells per 140 minutes			
		A	B	C	D
		uL	uL	uL	uL
0.25 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose		96.0	146.0	93.6	87.9
		90.2	128.8	101.4	87.3
	Ave.	93.1	137.4	97.5	87.6
1.0 ml. of chromatogram extract		60.3	97.5	58.1	57.5
		59.3	101.6	60.5	56.0
	Ave.	59.8	99.6	59.3	56.8
1.0 ml. of chromatogram extract with 0.5 ml. of 0.1 M. glucose		91.7	136.2	85.2	82.6
		85.0	124.0	92.1	83.6
	Ave.	88.4	130.1	88.7	83.2

*Suspensions were diluted to 10 per cent transmission. Dry weights of suspensions 1, 2, 3 and 4 were 3.46, 4.68, 6.01 and 4.55 mg. per ml., respectively.

**The results obtained with these suspensions of cells apparently are due to an error that may have resulted from glucose being added to the "treated paper control" flask instead of to the "glucose" flask.

TABLE 35

RESPIRATION MEASUREMENTS ON 48 HOUR PEPTONE-YEAST EXTRACT CELL SUSPENSIONS*
USING CHROMATOGRAPHED CELL EXTRACT ADDED AS SUBSTRATE

Substrate	Suspension:	Oxygen absorbed per mg. of cells per 140 minutes 1	2	3	4
		uL	uL	uL	uL
None (endogenous values)		31.5	38.3	36.6	50.9
		29.2	31.9	34.4	50.6
	Ave.	30.4	35.1	35.5	50.8
1.0 ml. of treated paper control extract		35.9	41.9	39.4	49.7
		37.5	44.7	46.6	47.6
	Ave.	36.7	43.3	43.0	48.7
0.5 ml. of 0.1 M. glucose		76.5	86.0	80.4	90.9
		75.6	84.3	79.8	82.5
	Ave.	76.1	85.2	80.1	86.7
0.5 ml. of 0.1 M. glucose with 1.0 ml. of treated paper control extract		80.7	92.5	81.4	98.0
		81.1	92.2	87.5	100.1
	Ave.	80.9	92.4	84.5	99.1
	With Chromatogram Extract:	A	B	C	D
0.25 ml. of chromatographed extract		28.3	34.9	38.0	48.2
		31.1	35.2	39.3	48.6
	Ave.	29.7	35.1	38.7	48.4

(Table continued on following page)

TABLE 35 (continued)

Substrate	With chromatogram extract:	Oxygen absorbed per mg. of cells per 1h0 minutes A	B	C	D
		uL	uL	uL	uL
0.25 ml. of chromatogram extract with		7h.8	86.6	78.2	89.9
0.5 ml. of 0.1 M. glucose		72.5	83.0	79.6	----**
	Ave.	73.7	84.8	78.9	
1.0 ml. of chromatogram extract		33.h	h3.h	39.7	57.3
		3h.2	h2.5	39.6	h9.8
	Ave.	33.8	h3.0	39.7	53.6
1.0 ml. of chromatogram extract with		69.6	85.8	76.0	60.3
0.5 ml. of 0.1 M. glucose		72.6	88.6	76.9	----**
	Ave.	71.5	87.2	76.5	

*Suspensions were diluted to 10 per cent transmission. Dry weights of suspensions 1, 2, 3 and h were h.00, 5.06, h.72 and 3.89 mg. per ml., respectively.

**Sample was discarded because of a leak in the connections.

a concentration for appreciable effects; activity of the whole extracts depending upon combinations of substances that were separated during the chromatographing; and active substances may have been destroyed during the separations. Combinations of the offered explanations might also account for the lack of effect of the fractions.

The values noted in Table 34 as possible errors may have resulted from glucose being added to the "treated paper control extract" flask and not being added to the "glucose" flasks. This would obviously give high values for the "treated paper control extract" flasks. This manipulative error would cause the "glucose" flasks to be like "endogenous" flasks except that the total volume would be 0.5 ml. less. This would account for the values shown by the "glucose" flasks being slightly higher than those for the "endogenous" flasks.

IV. SUMMARY AND CONCLUSIONS

The growth of A. gossypii occurs in small clumps which makes the preparation of a uniform suspension for respiration measurements difficult. Homogenization for 10 seconds in a cooled Waring blendor and shaking the final suspension with glass beads gave suspensions on which good duplication of results could be obtained.

Several factors, including the position of the thermobarometer on the water bath, the rate of shaking in the Warburg, the concentration of glucose used as substrate, the concentration of inoculum and the effect of cold storage were investigated in order to determine the optimal conditions for respiration measurements on the cell suspensions.

Using suspensions of 24, 48, 72 and 96 hour cells from four different media- peptone, glutamic acid-arginine, glutamic acid-arginine-leucine, and glutamic acid-arginine-leucine-histidine- it was found that the actively metabolizing cells had a higher respiration than the older cells when growth was normal. With the last two media, however, an inhibition or delay of growth occurred and cells from these media showed peaks in respiration at 24 and 48 hours, respectively. It was postulated that these peaks may be associated with the synthesis of substances necessary for riboflavin formation, since these peaks occurred during slow growth.

The addition of copper and iron to a glutamic acid-arginine-glycine medium gave cells with a respiration almost twice that of cells

of the same age grown on the same medium without copper or iron. This may be due to the increased activity of copper and/or iron containing enzymes.

The addition of riboflavin to peptone cells showed almost the same increase in respiration as did the addition of glucose in the concentrations studied. If this effect is due to oxidation of riboflavin instead of stimulation of endogenous respiration it may explain why riboflavin does not usually appear in appreciable amounts in non-inhibiting growth media until after the rapid growth phase.

Respiration measurements made on peptone, glutamic acid-arginine and glutamic acid-arginine-glycine cells using various nitrogen sources as substrates showed small effects in general. Of several purines used xanthine showed the largest increases in respiration on all three types of media, with guanine a fairly close second. If these effects are related to riboflavin formation, this would be in agreement with postulated purine pathways for synthesis of riboflavin. Various amino acids were run as substrates with these same media at pH 6.0 and at pH 8.0. at pH 6.0 only proline, alpha-alanine and glycine, of the amino acids used showed an effect proportional to the growth of cells in each of these media. Most of the DL amino acids used, except serine and alpha-alanine, gave only small increases in respiration possibly due to inhibition by the D forms. The respiration effects obtained with serine, glutamic acid, asparagine and tryptophan indicated a possible relationship to the formation of riboflavin. At pH 8.0, the respiration measurements were much lower, in general, than at pH 6.0. Very few of the amino acids used showed any stimulation of respiration in the presence of

glucose. Glycine, alpha-alanine and proline showed the same relative effects at pH 8.0 as at pH 6.0. Glutamic acid showed the largest effects and may be related indirectly to riboflavin formation. It appears to be related directly to growth.

The addition of water and acetone extracts of cells to peptone-yeast extract cells gave very large increases in the endogenous respiration and small increases in respiration in the presence of glucose. This increase in respiration appeared to be related to stimulation of endogenous respiration rather than to oxidation of substances present in the extracts. Chromatographed portions of the water extract, run with peptone-yeast extract cells, showed very slight effects on respiration. This may have been due to too great dilution or, perhaps, to a destruction or separation of active components during the chromatographing.

On a basis of findings in this investigation, further work would seem to be indicated on the function of glycine, serine, glutamic acid, arginine, tryptophan, leucine, histidine and xanthine as possible intermediates or starting compounds in the synthesis of riboflavin.

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BIOGRAPHICAL ITEMS

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